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The cell factory Ralstonia eutropha: Development of plasmid-based expression systems and analysis of the regulation of carbon dioxide fixation

DOCTORAL THESIS

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AFFIDAVIT

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Abstract

The Gram-negative, facultative chemolithoautotrophic bacterium *Ralstonia eutropha* H16 is a strictly respiratory prokaryote, which can use H_2 and CO_2 as sole energy and carbon sources in absence of organic substrates. The use of *R. eutropha* H16 as a production organism attracted significant interest based on its ability to grow under lithoautotrophic conditions, to produce large amounts of the biodegradable polymer polyhydroxyalkanoates (PHA) and the capability to grow to high cell densities. However, a comprehensive toolbox including suitable inducible expression systems, stably maintained plasmid vectors or the ability to promote protein secretion has not yet been established. Consequently, this work aimed to create a set of stably maintained and versatile plasmid vectors for the use in *R. eutropha* H16 under lithoautotrophic or heterotrophic growth conditions based on homologous and heterologous building blocks. The focus was set on the characterization of suitable minireplicons, elements promoting plasmid stability, promoter sequences, secretion signal sequences and the construction of inducible expression systems. Altogether, a set of plasmid vectors was constructed on the basis of pSa, RP4, RSF1010 and pBBR derived minireplicons that were significantly stabilized by the RP4 *par* region encoding a toxin/antidote system, a plasmid multimer resolution system and a plasmid segregation system. Moreover, a set of highly active promoters and two inducible expression systems based on the lac and cumate regulatory elements could be successfully established, which enable highly tunable and tightly regulated expression of the gene of interest in *R. eutropha* H16.

In a second approach, the transcription regulation of the two *cbb* operons encoded in the genome of *R. eutropha* H16 was analyzed in more detail. It could be established that the activity of both *cbb* promoters is dependent on the transcription regulator RegA as part of the global transcription regulation system RegA/RegB, next to the main transcription regulator CbbR. According to this, the CbbR-based transcription regulation is thought to represent a feedback control based on the carbon-state of the cell while the RegA-based control depends on the cellular energy-state.

Kurzfassung

Ralstonia eutropha H16 ist ein Gram-negatives, fakultativ chemolithoautotrophes Bakterium das H_2 und CO_2 in der Abwesenheit von organischen Substraten als einzige Energie und Kohlenstoffquelle verwerten kann. Die biotechnologische Nutzung dieses Bakteriums ist von großem Interesse, da *R. eutropha* H16 unter lithoautotrophen Bedingungen wächst, große Mengen des natürlich abbaubaren Polymers Polyhydroxyalkanoat produzieren kann und zu sehr hohen Zelldichten anwachsen kann. Um *R. eutropha* H16 als Produktionsorganismus für Proteine und Metabolite nutzen zu können werden unter anderem stabile Expressionsvektoren, induzierbare Expressionssysteme oder die Möglichkeit zur Protein Sekretion benötigt. Das Ziel dieser Arbeit war es, ein Set von stabilen und vielseitig einsetzbaren Expressionsplasmiden zu entwickeln und die biotechnologische Nutzung von *R. eutropha* H16 unter lithoautotrophen und heterotrophen Wachstumsbedingungen zu ermöglichen. Wichtige Elemente für die Konstruktion der Expressionsplasmide waren Replikationselemente unterschiedlicher Plasmide, Systeme zur Steigerung der Plasmidstabilität, Promotoren, Signalsequenzen zur Proteinsekretion und induzierbare Expressionssysteme. Basierend auf den Replikationselementen der Plasmide pSa, RP4, RSF1010 und pBBR1 wurden Expressionsplasmide konstruiert, welche mittels der RP4 *par* Sequenz wesentlich stabilisiert wurden. Des Weiteren wurden mehrere hochaktive Promotoren und zwei induzierbare Expressionssysteme, basierend auf Lac und Cumate Regulationselementen, konstruiert und charakterisiert. Somit konnte ein Set von äußerst stabilen und vielseitig einsetzbaren Expressionsplasmiden geschaffen werden, welches die gewünschte Expressionsregulation des Zielgens unter lithoautotrophen und heterotrophen Wachstumsbedingungen ermöglichte.

In einem weiteren Ansatz, wurde die Transkriptionsregulation des *cbb* Operons in *R. eutropha* H16 eingehend untersucht. Dabei konnte gezeigt werden, dass die Aktivität beider *cbb* Promotoren nicht nur von dem bereits bekannten Transkriptionsfaktor CbbR beeinflusst wurde, sondern auch von RegA, einem Transkriptionsfaktor des globalen Transkriptionsregulationssystems RegA/RegB. Die CbbR abhängige Transkriptionskontrolle der *cbb* Promotoren scheint hierbei ein zelluläres Feedback des Kohlenstoffmetabolismus der Zelle zu sein und die Transkriptionskontrolle von RegA ein Feedback des Energiemetabolismus der Zelle.

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Abbreviations

ADP	adenosine diphosphate
Ap^r	ampicillin resistance
ATP	adenosine triphosphate
bp	base pair(s)
CBB	Calvin-Benson-Bassham cycle
CDS	coding sequence
CoA	coenzyme A
cumate	4-Isopropylbenzoic acid
CymR	gene encoding cumate repressor
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
E. coli	*Escherichia coli*
ED	Entner-Doudoroff pathway
EGFP	enhanced green fluorescent protein
EstA	Esterase A
FAD	flavin adenine dinucleotide
CFU	colony forming unit
Gm	gentamicin
His	histidine
IPTG	isopropyl-β-D-thiogalactopyranoside
KDPG	2-keto-3-deoxy-6-phosphogluconate
Km	kanamycin
kDa	kilodalton
lacI	gene encoding *lac* repressor
LacZ	β-galactosidase
LB	lysogeny broth
MBH	membrane–bound hydrogenase

M-FDH	membrane bound formate dehydrogenase
mob pBBR1	mobilization region of pBBR1-MCS5 plasmid
mob RP4	mobilization region of RP4 plasmid
mob RSF1010	mobilization region of RSF1010 plasmid
MQ	menaquinone
MU	Miller Units
NaCl	sodium chloride
NAD(H)	nicotineamid adenine dinucleotide (reduced form)
NADP(H)	nicotineamid adenine dinucleotide phosphate (reduced form)
NaOH	sodium hydroxide
NAR	nitrate reductase
NIR	nitrite reductase
NOR	nitric oxide reductase
NOS	nitrous oxide reductase
OD_{600}	optical density at 600 nm
ONC	overnight culture
par	RP4 *par* region
PCR	polymerase chain reaction
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate
PHBV	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
rpm	revolutions per minute
Rubisco	ribulose-1,5-biphosphate carboxylase/oxygenase
R. eutropha H16	*Ralstonia eutropha* H16
RH	regulatory hydrogenase
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S-FDH	soluble formate dehydrogenase
SH	soluble hydrogenase

TCA	tricarboxylic acid cycle
TSB	tryptic soy broth
U	enzyme activity unit
UQ	ubiquinone
UV	ultraviolet light

1. Introduction

1.1 *Ralstonia eutropha* H16

Ralstonia eutropha H16 (now known as *Cupriavidus necator*) is a soil-dwelling, facultative lithoautotrophic Gram-negative β-proteobacteria of the *Burkholderiales* order (Bowien & Kusian, 2002; Schwartz *et al.*, 2009). Like many other β-proteobacteria *R. eutropha* H16 carries a multi replicon genome, which is comprised of two chromosomes and one megaplasmid (pHG1) (Pohlmann *et al.*, 2006). The majority of housekeeping genes including functions related to replication, translation or transcription is encoded on chromosome 1 (4052032 bp), whereas alternative metabolic pathways enabling for example the use of a variety of carbon sources are located on chromosome 2 (2912490 bp) (Pohlmann *et al.*, 2006). The genetic information present on the megaplasmid (452156 bp) was found to code for metabolic features such as autotrophic carbon dioxide fixation, hydrogen oxidation or denitrification (Pohlmann *et al.*, 2006; Schwartz *et al.*, 2003). Naturally growing at the interface of aerobic and anaerobic environments the metabolism of *R. eutropha* H16 is well adapted to changing ambient conditions with respect to energy and carbon sources. Accordingly, the bacterium can easily adopt a heterotrophic or autotrophic lifestyle and is capable to perform aerobic or anaerobic respiration (Bowien & Kusian, 2002; Cramm, 2009). Accepted carbon and energy sources under heterotrophic growth conditions include a versatile range of numerous simple organic acids and sugars like fructose or N-acetylglucosamine, which are metabolized via the Entner–Doudoroff pathway and the TCA cycle (Figure 1 and 2) (Cramm, 2009).

In the absence of such compounds, *R. eutropha* H16 is able to grow autotrophically by fixing CO_2 via the Calvin-Benson-Bassham cycle (CBB), including ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) type I as the CO_2 fixing enzyme, and oxidizing hydrogen to provide the cell with energy (Figure 1 and 2) (Bowien & Kusian, 2002). The oxidation of hydrogen does also serve as an energy source under a broad range of growth conditions based on the CO and O_2 tolerance of [NiFe]-hydrogenases (Burgdorf *et al.*, 2005). Next to the ability to grow under heterotrophic or autotrophic conditions in the presence of oxygen, *R. eutropha* H16 is also able to utilize alternative electron receptors to perform respiration under anoxic conditions. Consequently, *R. eutropha* H16 can use nitrate or nitrite as electron receptors and respire these to nitrogen. This is enabled by a cluster of oxidoreductases responsible for the process of denitrification encoded on chromosome two and pHG1 (Kohlmann *et al.*, 2014).

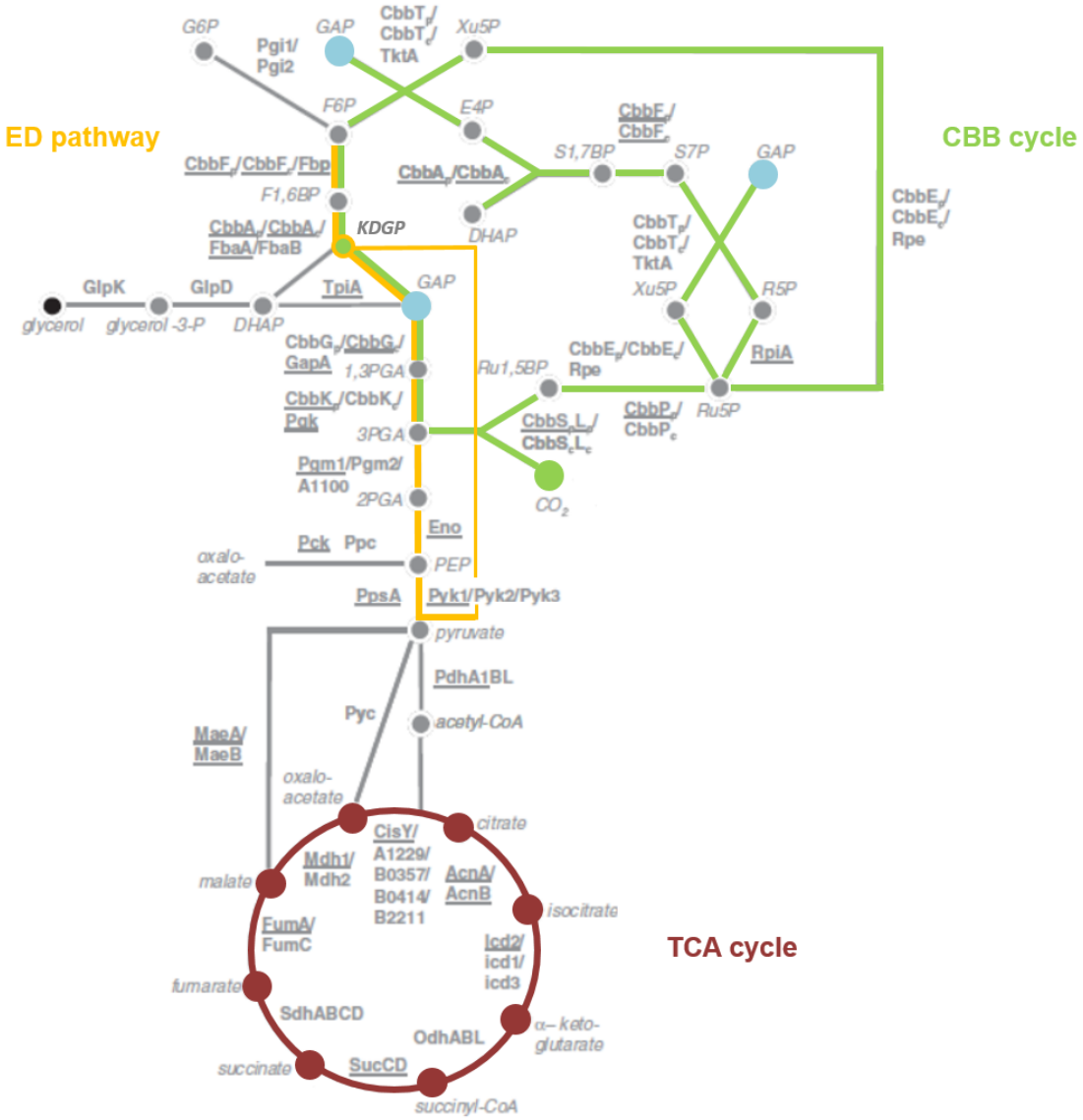


Figure 1: Central carbon metabolism of *R. eutropha* H16. The enzyme, or the loci of annotated enzymes, and the metabolites of the carbon metabolism of *R. eutropha* H16 are shown in grey. The Entner–Doudoroff (ED) pathway is indicated in orange, the Calvin-Benson-Bassham (CBB) cycle and the regenerating reactions are shown in green. The tricarboxylic acid (TCA) cycle is labelled in red. Image adapted from Schwartz *et al.* (2009).

The bacterium does also possess the ability to accumulate large quantities of polyhydroxybutyrate (PHB) as a natural carbon and energy storage under growth limiting conditions (Figure 2) (Schubert *et al.*, 1988; Steinbüchel & Füchtenbusch, 1998). PHB is stored as intracellular granules and can

account for as much as 90% of dry cell weight (Atlić *et al.*, 2011). The interest in PHB, based on its characteristics to serve as an alternative for some petroleum based polymers, has consequently increased the interest in *R. eutropha* H16 and its PHB producing properties (Atlić *et al.*, 2011; Steinbüchel & Füchtenbusch, 1998). However, especially the production of polyhydroxyalkanoates (PHA) that consist of short chain length (SLC) monomers, containing three to five carbon atoms, and medium chain length (MLC) monomers, six or more carbon atoms, prove to be more suitable for replacing a larger number of petroleum-based polymers than PHB (Luengo *et al.*, 2003; Noda *et al.*, 2005a). Since PHB does only consist of SLC monomers, specifically 3-hydroxybutyrate, it is more complex to process and has a lower flexibility than PHA copolymers consisting of SLC and MLC (Noda *et al.*, 2005a; Wang *et al.*, 2013).

Moreover, the MLC content does significantly influence the properties of PHA copolymers related to crystallinity or the melting temperature (Noda *et al.*, 2005b). Consequently, *R. eutropha* H16 strains were engineered for the production of various PHAs such as the copolymer poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) or (P(HB-co-HHx), which shares similar properties with low-density polyethylene (LDPE) when HHx is present in high amounts of the (HB-co-HHx) polymer (Doi *et al.*, 1995). In this particular case, P(HB-co-HHx) was produced in recombinant *R. eutropha* H16 strains that were engineered to express heterologous PHA synthases and other PHA synthesis related enzymes (Budde *et al.*, 2011). This approach exemplifies the majority of strain engineering performed on the basis of *R. eutropha* H16, which aimed to take advantage of the organism's ability to produce large amounts of the desired polymer and to grow to high cell densities (Budde *et al.*, 2011; Kim *et al.*, 2005; Luengo *et al.*, 2003; Wang *et al.*, 2013). This includes next to a large number of different PHA copolymers, the amino acid based polymer cyanophycin, which is composed of an aspartic acid backbone and arginine side groups (Diniz *et al.*, 2006). Cyanophycin can be used as a source of polyaspartic acid, which in turn has potential to replace a number of polymers that are not biodegradable (Roweton *et al.*, 1997).

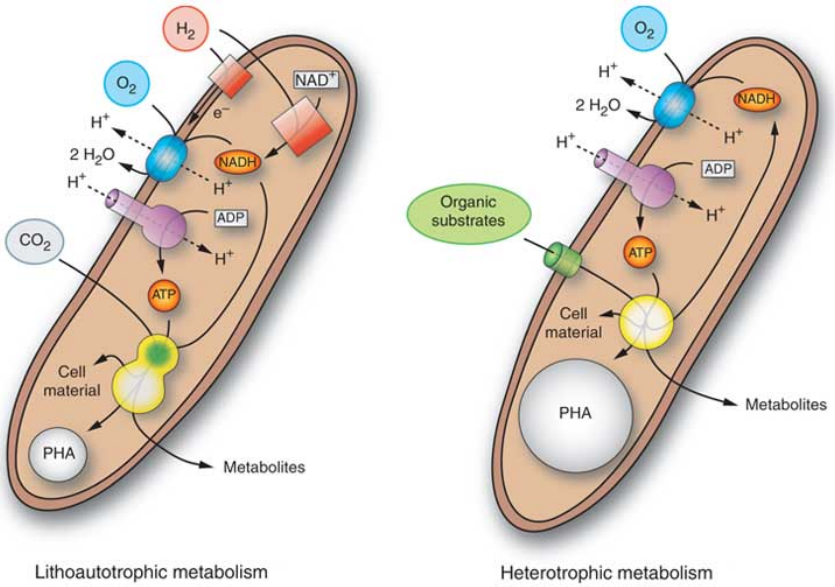


Figure 2: Schematic representation of the lithoautotrophic and heterotrophic metabolism of *R. eutropha* H16. Illustration of the essential metabolic pathways and key intermediates under lithoautotrophic or heterotrophic growth conditions of *R. eutropha* H16. Image taken from Pohlmann *et al.* (2006).

Next to the synthesis of a large number of biodegradable polymers, interest in *R. eutropha* H16 has also been increasing with respect to the production of biotechnologically relevant proteins and metabolites under lithoautotrophic as well as heterotrophic growth conditions (Barnard *et al.*, 2004; Diniz *et al.*, 2006; Lutte *et al.*, 2012). One such example is the synthesis of isotope-labelled arginine in *R. eutropha* H16 under lithoautotrophic conditions, using $^{13}CO_2$ and H_2 as sole carbon and energy sources, respectively (Lutte *et al.*, 2012). Yet in another case, *R. eutropha* H16 was successfully engineered to produce significant amounts of 2-methylcitric acid under heterotrophic conditions (Ewering *et al.*, 2006). However, especially the ability of *R. eutropha* H16 to produce large amounts of properly folded protein under stress conditions with no significant inclusion body formation represents a promising feature for further strain engineering with respect to biotechnological applications (Gruber *et al.*, 2014; Srinivasan *et al.*, 2002). Expression of the enzyme organophosphate hydrolase (OPH) in *R. eutropha* H16 did for example result in the formation of large amounts of active and soluble enzyme, unlike the production of OPH in *E. coli* cultures, which resulted predominantly in the accumulation of inclusion bodies (Barnard *et al.*, 2004; Srinivasan *et al.*, 2002).

The formation of active OPH does most likely relate to a different redox dependent intracellular environment and different codon usage compared to *E. coli*, which in turn appears to be beneficial for the formation of numerous other proteins (Gruber *et al.*, 2014; Hess *et al.*, 2015; Srinivasan *et al.*, 2002).

Another advantage for the application of *R. eutropha* H16 as a production host is the ability of the bacterium to grow to high cell densities without accumulating growth inhibiting organic acids (Barnard *et al.*, 2004). As a consequence, high cell densities can be accomplished with *R. eutropha* H16 as production host and enable a fermentation process that provides higher product concentrations, increased productivity and improved operating costs (Andersson *et al.*, 1994; Chen *et al.*, 1992). Accordingly, large scale high-cell-density fermentations on the basis of *R. eutropha* H16 yielded large amounts of the target protein and high cell densities of 230 g/l (Barnard *et al.*, 2004; Ryu *et al.*, 1997). Moreover, the versatility of *R. eutropha* H16 to accept a wide range of carbon and energy sources for the production of value-added products also enables the use of waste products or renewable resources as growth substrates, including for example waste water, whey, molasses, various plant oils, low quality waste animal fat, formate or CO_2 and H_2 (Budde *et al.*, 2011; Grunwald *et al.*, 2015; Huschner *et al.*, 2015; Lutte *et al.*, 2012; Oliveira *et al.*, 2004; Riedel *et al.*, 2015). In this context, the use of CO_2 or formate, which can easily be created on an electrochemical basis from CO_2 (Li *et al.*, 2012), is unique with respect to strain engineering and fermentation processes. Several attempts have been made to divert carbon-flux towards the production of value-added products in *R. eutropha* H16 using CO_2 or formate as sole carbon sources. This includes amongst others the production of isotope-labelled arginine, PHB, methyl ketones, or isobutanol (Brigham *et al.*, 2013; Garcia-Gonzalez *et al.*, 2015; Grunwald *et al.*, 2015; Islam Mozumder *et al.*, 2015; Lutte *et al.*, 2012; Müller *et al.*, 2013).

1.2 Carbon and energy metabolism of *R. eutropha* H16

1.2.1 Heterotrophic metabolism of *R. eutropha* H16

In the presence of oxygen and suitable carbon sources *R. eutropha* H16 adopts a heterotrophic lifestyle. Carbon and energy sources under these conditions include a variety of simple organic acids, fatty acids, aromatic compounds and hexose sugars like fructose or *N*-acetylglucosamine, which are metabolized via the Entner–Doudoroff (ED) pathway and the TCA cycle (Budde *et al.*, 2011; Cramm, 2009; Pohlmann *et al.*, 2006). The utilization of sugars by *R. eutropha* H16 is limited to fructose and *N*-acetylglucosamine since membrane transport proteins for other saccharides like glucose or lactose are absent and key enzymes of the Embden-Meyerhof-Parnas (EMP) pathway as well as the oxidative pentose phosphate pathway, namely phosphofructokinase and 6-phosphogluconate dehydrogenase, are not encoded on the genome (Cramm, 2009; König *et al.*, 1969). The transport of *N*-acetylglucosamine across the membrane of *R. eutropha* H16 the is likely to be carried out by phosphotransferase-type transport system, while the transport of fructose is most probably mediated by an ABC transporter (Pohlmann *et al.*, 2006). Fructose is then catabolized via the ED pathway which involves the cleavage of the key intermediate 2-keto-3-deoxy-6-phosphogluconate (KDPG) by KDPG aldolase to glyceraldehyde-3-phosphate (GAP) and pyruvate (Conway, 1992; Cramm, 2009). GAP is further catabolized in a sequence of steps to yield pyruvate as well. Pyruvate is then fed into the TCA cycle to generate NADH and ATP. NADH generated by the TCA cycle is used in the oxidative phosphorylation pathway to generate more ATP (Conway, 1992; Pohlmann *et al.*, 2006).

The use of the ED pathway in *R. eutropha* H16 and many other prokaryotes is thought to strongly depend on the amount of enzyme protein necessary to maintain the pathway's flux and its energy yield (ATP) (Flamholz *et al.*, 2013). However, the ED pathway generates only half the ATP compared to the EMP pathway at the same rate of glucose conversion, 1 ATP, 1 NADH and 1 NADPH per molecule of glucose are generated by the ED pathway in comparison to 2 ATP and 2 NADH per molecule of glucose by the EMP pathway (Bar-Even *et al.*, 2012; Conway, 1992; Fuhrer *et al.*, 2005). Even though the ED pathway generates less ATP from one molecule of glucose, it requires substantially less enzymatic protein to maintain the pathway's flux (Bar-Even *et al.*, 2012; Flamholz *et al.*, 2013). It is thought that *R. eutropha* H16, such as many other prokaryotes, is able to generate sufficient ATP through non-glycolytic energy sources and perform glucose conversion at the same rate with a lower ATP yield on the basis of the ED pathway, but save recourses by

requiring substantially less enzyme protein for the overall process (Flamholz *et al.*, 2013; Fuhrer *et al.*, 2005). Accordingly, a pattern has been observed supporting the assumption that the ED pathway predominates in prokaryotes with the ability to access sufficient non-glycolytic sources of ATP allowing to save resources by synthesizing less amounts of glycolytic enzymes (Flamholz *et al.*, 2013).

1.2.2 Lithoautotrophic and organoautotrophic metabolism of *R. eutropha* H16

The ability to grow lithoautotrophically enables *R. eutropha* H16 to utilize CO_2 and molecular hydrogen as sole carbon and energy source, respectively (Bowien & Kusian, 2002). Lithoautotrophic growth of *R. eutropha* H16 is facilitated by hydrogen oxidation carried out by hydrogenases and carbon dioxide fixation via the enzymes of the CBB cycle (Bowien & Kusian, 2002; Pohlmann *et al.*, 2006). The oxidation of hydrogen is mediated by three different hydrogenases, namely a regulatory hydrogenase (RH), a membrane bound hydrogenase (MBH) and a soluble hydrogenase (SH) (Burgdorf *et al.*, 2005). All hydrogenases found in *R. eutropha* H16 belong to the [Ni-Fe] family of hydrogenases (Burgdorf *et al.*, 2005; Schwartz *et al.*, 2003).

Hydrogen oxidation

Altogether, three different groups of hydrogenase metalloenzymes are found in nature, which are categorized according to the composition of their active site including the [Fe] hydrogenases, [Fe-Fe] hydrogenases and [Ni-Fe] hydrogenases (Corr & Murphy, 2011; Fontecilla-Camps *et al.*, 2007). Among these, [Fe-Fe] hydrogenases and [Ni-Fe] hydrogenases are the most common hydrogenases found in nature occurring mainly in bacteria and archaea species. The majority of enzymes in both groups of hydrogenases are active in microaerobic and anaerobic environments, but are inactivated by higher oxygen concentrations (Burgdorf *et al.*, 2005; Fontecilla-Camps *et al.*, 2007). However, a sub-group of [Ni-Fe] hydrogenases identified in *R. eutropha* H16 is remarkably tolerant to oxygen and carbon monoxide (Burgdorf *et al.*, 2005; Lenz *et al.*, 2010). The oxygen tolerance of these [Ni-Fe] hydrogenases is mainly based on the unique architecture of the active centre. In comparison to [Fe-Fe] hydrogenases, [Ni-Fe] hydrogenases have a unique Cys_6[4Fe–3S] centre located closely to the active [Ni-Fe] centre allowing for H_2 oxidation in the presence of oxygen (Shomura *et al.*, 2011). Furthermore, the activity of the enzymes is biased towards H_2 oxidation compared to proton reduction and [Ni-Fe] hydrogenases possess a mechanism that allows temporarily O_2-inhibited

enzymes to be reactivated (Fritsch *et al.*, 2013; Lukey *et al.*, 2010). This enables *R. eutropha* H16 to use H_2 as an energy source under various growth conditions.

All genes coding for [Ni-Fe] hydrogenases in *R. eutropha* H16 are present in the *hox* operon located on the megaplasmid (Schwartz *et al.*, 2003). Transcription of the *hox* operon is controlled by the histidine sensor kinase HoxJ, an NtrC type transcription activator (HoxA) and RH. In the absence of hydrogen HoxJ is dissociated from RH and HoxJ remains active phosphorylating HoxA. The phosphorylation of HoxA in turn results in the inactivation of its function as a transcriptional activator (Lenz *et al.*, 2010). However, in the presence of hydrogen RH oxidizes H_2 at a very low turnover rate and forms a complex with HoxJ. This inhibits the kinase activity of HoxJ and leaves HoxA unphosphorylated in its active form (Lenz *et al.*, 2010). Consequently, HoxA is able to activate transcription of the *hox* operon by recruiting RNA polymerase at the *hox* promoter involving the sigma factor σ^{54} (Friedrich *et al.*, 2005).

The *hox* operon encodes genes for the hydrogenases SH and MBH. SH is a multimeric hydrogenase which is found in the cytoplasm where it catalyses the oxidation of hydrogen and reduction of NAD+ to NADH (Fritsch *et al.*, 2013). The MBH genes encode a heterodimeric hydrogenase that is membrane-bound via a *b*-type cytochrome. The formation of the MBH involves a maturation process with several steps of proteolytic processing and complex formation (Lenz *et al.*, 2010). The mature MBH consists of a membrane anchor (HoxZ), the catalytic subunits (HoxG) including the hydrogen splitting Ni-Fe active center and an electron transfer subunit (HoxK). The electrons are transported from the active center of HoxG via Fe-S clusters in HoxK and heme groups in HoxZ to ubiquinone (Bernhard *et al.*, 1997; Fritsch *et al.*, 2013).

Autotrophic growth and Carbonic anhydrases

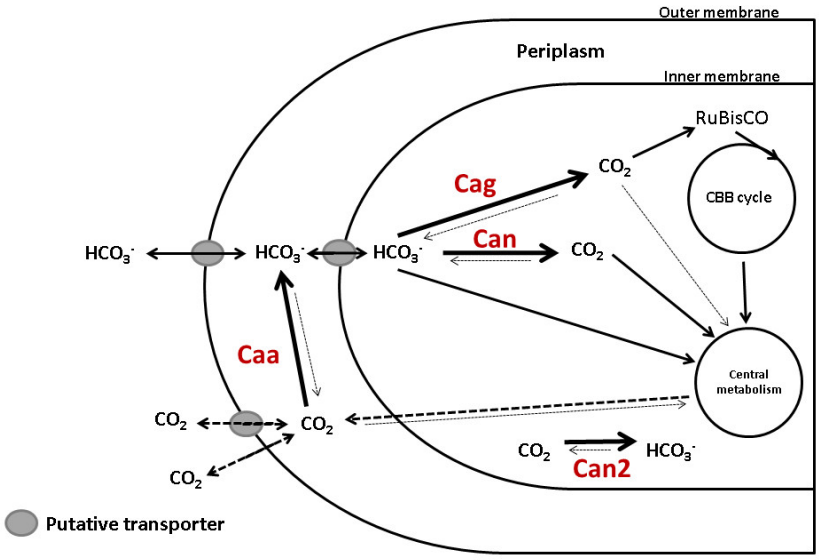


Figure 3: Function of carbonic anhydrases in *R. eutropha* H16. Caa converts CO_2 to HCO_3^- to supply the cell's metabolism, Can and Cag provide CO_2 to the CBB cycle. Can2 is involved in pH maintenance. Image taken from Gai *et al.* (2014).

Autotrophic growth of *R. eutropha* H16 under atmospheric conditions depends on the presence of carbonic anhydrases in order to control intracellular pH and to provide CO_2 concentrations sufficient for carbon fixation (Codd & Kuenen, 1987; Gai *et al.*, 2014). In total four genes encoding carbonic anhydrases were identified on the genome of *R. eutropha* H16 including *can* (*H16_A0169*), *can2* (*H16_B2270*), *caa* (*H16_B2403*) and *cag* (*H16_A1192*). These genes were found to encode three different types of carbonic anhydrases, *can* and *can2* encode β-carbonic anhydrases, *caa* codes for a periplasmic α-carbonic anhydrase and *cag* represents a γ-like carbonic anhydrase (Gai *et al.*, 2014; Kusian *et al.*, 2002). All carbonic anhydrases are thought to obtain different functions; the periplasmic Caa converts CO_2 to HCO_3^- to supply the cell's metabolism with HCO_3^-, Can and most likely also Cag primarily supply CO_2 for carbon fixation to Rubisco, while Can2 is involved in pH maintenance (Figure 3) (Gai *et al.*, 2014; Kusian *et al.*, 2002).

CO_2 fixation by the CBB-cycle

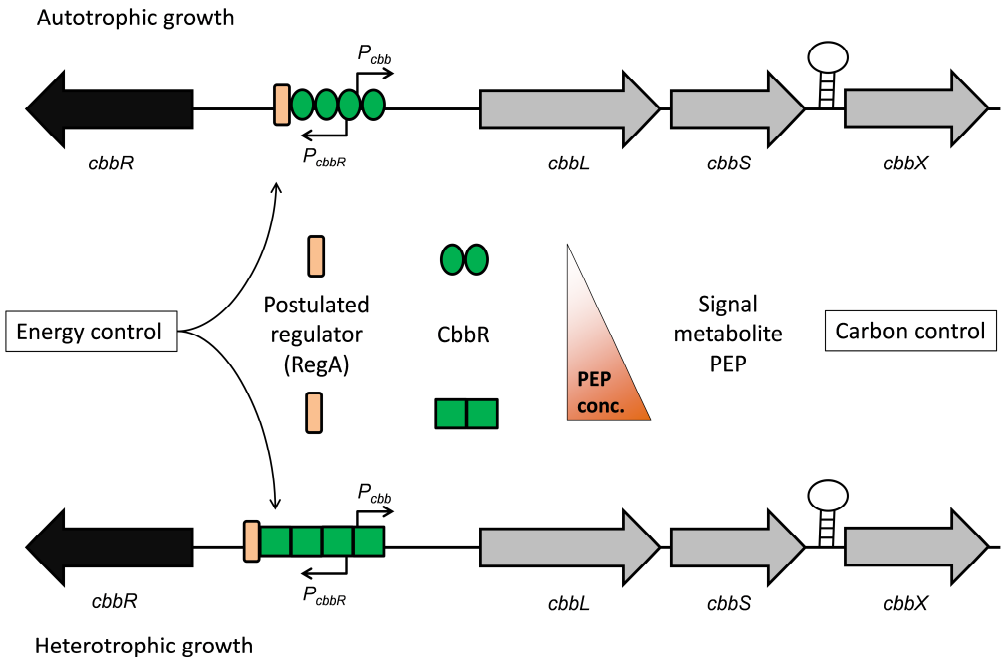


Figure 4: Transcription regulation of the *cbb* operon in *R. eutropha* H16 under heterotrophic and autotrophic growth conditions. The transcription regulator CbbR controls the activity of P_{cbb} dependent on cellular PEP levels. This is expected to represent a feedback control based on the carbon-state of the cell. High PEP levels repress transcription (under heterotrophic growth conditions), while low PEP levels promote expression (under autotrophic growth conditions). Furthermore, an additional feedback control is thought to influence *cbb* operon transcription reflecting the energy-state of the cell. Image adapted from Bowien & Kusian (2002).

Carbon dioxide is the main carbon source for *R. eutropha* H16 under lithoautotrophic growth conditions and is assimilated by the enzymes of the Calvin-Benson-Bassham (CBB) cycle. All CBB-related enzymes are encoded on the genome of *R. eutropha* H16 in duplicate, one *cbb* operon is located on chromosome two and an almost identical copy on the megaplasmid (Bowien & Kusian, 2002; Pohlmann *et al.*, 2006). A high degree of homology is shared on a nucleotide level by the two *cbb* operons including an identical arrangement of the particular *cbb* promoters, CbbR binding sites and a similar number of genes (Bowien & Kusian, 2002). In comparison to the *cbb* operon located on the chromosome, the *cbb* operon on pHG1 lacks a gene coding for a formate dehydrogenase (*cbbB*) and a gene encoding the transcription regulator (*cbbR*). A highly homologous DNA sequence similar to *cbbR* can also be found on pHG1, but no functional product is formed due to the lack of a

complete open reading frame (Bowien & Kusian, 2002; Pohlmann *et al.*, 2006). The transcription of both *cbb* operons is driven by a σ^{70} promoter (P_{cbb}), which is located directly upstream of $cbbL_{C,P}$, and the relative abundance of *cbb* gene transcripts is influenced by an mRNA based stem-loop. This loop forms in the intergenic region of *cbbS* and *cbbX* causing a difference in gene expression levels within the *cbb* operons (Bowien & Kusian, 2002; Schäferjohann *et al.*, 1996). However, the main transcription regulation of the *cbb* operons is executed by CbbR, a LysR-type transcriptional regulator (LTTR) that binds as a tetramer upstream of P_{cbb}. CbbR binds to an activator and regulator binding site and represses *cbb* operon transcription depending on the presence of the signal metabolite phosphoenolpyruvate (PEP) (Figure 4) (Bowien & Kusian, 2002). This is thought to represent a feedback dependent on the carbon-state of the cell and represses *cbb* operon transcription in the presence of high PEP levels or activates transcription when cellular PEP levels are low (Bowien & Kusian, 2002; Jeffke *et al.*, 1999). However, the transcription of *cbb* operons is expected to be not only influenced by the carbon-state of the cell, but also by a mechanism that reflects the energy-state of the cell (Figure 4). The influence regarding an additional transcription regulation of both *cbb* operons in *R. eutropha* H16 with respect to the energy-state of the cell is further elaborated in chapter 4.

The CO_2 fixing enzyme ribulose-1,5-bisphosphate-carboxylase/-oxygenase (Rubisco) type I carries out the carboxylation reaction in *R. eutropha* H16, facilitating the assimilation of inorganic carbon (Kusian *et al.*, 1995). In general there are four different types of Rubisco enzymes found in nature, categorized as type I, II, III and IV depending on differences in the primary sequence (Tabita *et al.*, 2008). Altogether, these different types of Rubisco enzymes are found in most autotrophic organisms including archaea, bacteria or algae and higher plants. Even though the structural composition among the different types of Rubisco can differ significantly, all Rubisco enzymes share the same large, catalytic subunit dimer and need to be activated by carbamylation at a specific lysine residue (Schneider *et al.*, 1992; Tabita *et al.*, 2007). The type I Rubisco found in eukaryotes and bacteria is the most abundant form of Rubisco occurring in nature and can further be separated in a red and a green branch (Badger & Bek, 2008; Tabita *et al.*, 2008). The red-type I Rubisco, as found in bacteria such as *R. eutropha* H16, red algae and phytoplankton, has a higher CO_2/O_2 specificity compared to green-type I Rubisco proteins that are present in cyanobacteria, green algae and plants. Such as all type I Rubisco proteins, the red-type I Rubisco that is present in *R. eutropha* H16, is composed of eight small and large subunits (L_8S_8), respectively (Badger & Bek, 2008; Schneider *et al.*, 1992).

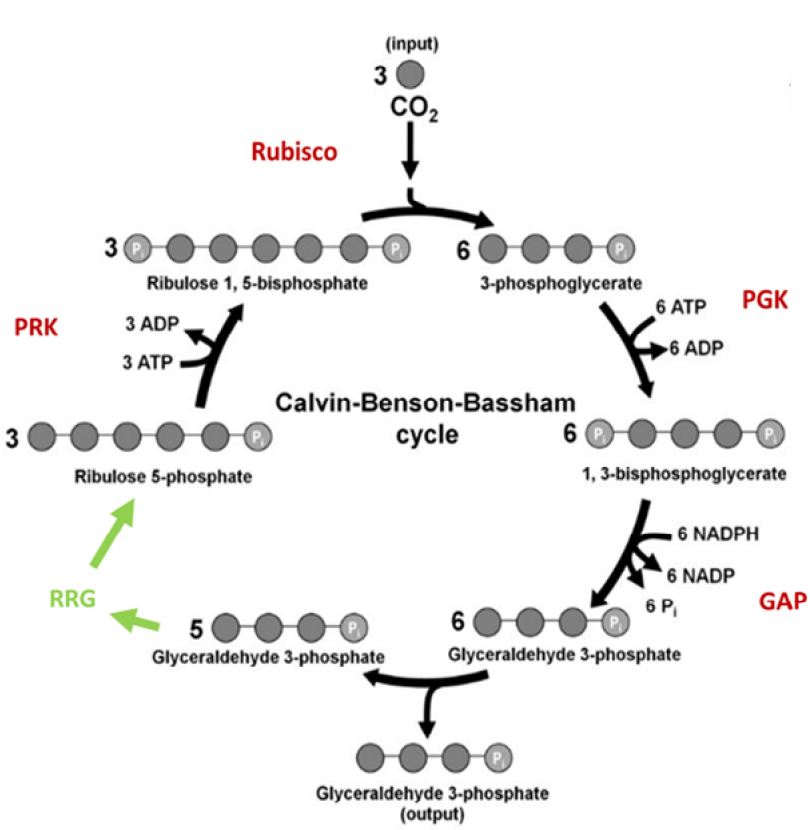


Figure 5: Reactions of the CBB cycle including carbon dioxide fixation. Illustration of the key intermediate metabolites of the CBB cycle and enzymes involved. Enzymes are shown in red: Rubisco, PGK (phosphoglycerate kinase), GAP (glyceraldehyde-3-phosphate dehydrogenase), PRK (phosphoribulose kinase). The multistep regeneration of ribulose-5-phopshate (RRG) is illustrated in detail in Figure 1. Overall reaction of the CBB cycle: $3CO_2$ + 6 NADPH + $5H_2O$ → Glyceraldehyde-3-phosphate + 6 NADP + 9 ADP + 8 P_i. Image adapted from Park *et al.* (2011).

In order for *R. eutropha* H16 to fix CO_2 and grow under lithoautotrophic conditions, Rubisco needs to maintain activity promoting the functionality of the CBB cycle. The catalytic activity of Rubisco depends on the cofactor Mg^{2+} and carbamylation of the enzyme by a "non-substrate" CO_2 (Cleland *et al.*, 1998; Mueller-Cajar *et al.*, 2011). Rubisco can then carry out the carboxylation of ribulose-1,5-bisphosphate (RuBP) thereby assimilating CO_2 (Figure 5). This reaction forms an unstable product, which immediately splits into two molecules of 3-phosphoglycerate (3-PG). The 3-PG molecules are further reduced to glycerinaldehyde-3-phosphate and are subsequently regenerated via different metabolic pathways in multiple steps involving enzymes such as triose phosphate isomerase, transketolases or transaldolases to yield ribulose-5-phosphate (Figure 1). In a final step, ribulose-5-phosphate is phosphorylated to RuBP by phosphoribulokinase (CbbP) (Figure 5) (Bowien & Kusian,

2002; Schwartz *et al.*, 2009). Altogether, the functionality of the entire CBB cycle does mainly rely on the presence of active Rubisco. The carbon fixing enzyme is, however, subject to significant inhibition caused for example by binding of xylulose-1,5-bisphosphate (XuBP) or RuBP to non-carbamylated Rubisco (Mueller-Cajar *et al.*, 2011; Parry *et al.*, 2008). Therefore, a number of proteins have evolved that are able to restore and maintain Rubisco activity. In *R. eutropha* H16 these proteins include CbbX, a AAA+ ATPase type protein, and CbbY, a XuBP phosphatase (Bracher *et al.*, 2015; Portis, 2003). Structural and functional studies conducted for a CbbX homologue found in *Rhodobacter sphaeroides* revealed that CbbX forms a hexameric ring that interacts with Rubisco to release inhibitory RuBP (Mueller-Cajar *et al.*, 2011). *CbbX* knock-out strains of *R. eutropha* H16 and *R. sphaeroides* lost the ability to grow autotrophically (Bowien & Kusian, 2002; Gibson & Tabita, 1997). CbbY is a sugar phosphatase that plays an important role in maintaining Rubisco activity. A side activity of Rubisco forms small amounts of XuBP, which is a potent inhibitor of Rubisco activity (Parry *et al.*, 2008). CbbY converts XuBP to xylulose-5-phosphate, which can be converted to RuBP (Bracher *et al.*, 2015; Pearce, 2006). However, *R. eutropha* H16 and *R. sphaeroides cbbY* knock-out strains were still able to grow under autotrophic conditions indicating a less severe inactivation of Rubisco activity by XuBP (Bowien & Kusian, 2002; Gibson & Tabita, 1997).

Organoautotrophic growth of *R. eutropha* H16

The ability of *R. eutropha* H16 to grow autotrophically is not limited to CO_2 as a substrate, but also includes the use of formate as an energy and carbon source (Grunwald *et al.*, 2015; Oh & Bowien, 1998). The organoautotrophic growth of *R. eutropha* H16 is promoted by formate dehydrogenases, which split formate into NADH and CO_2. The released CO_2 is in turn fixed by the CBB cycle (Oh & Bowien, 1998; Pohlmann *et al.*, 2006). A soluble and a membrane-bound formate dehydrogenase are formed in *R. eutropha* H16, but only the soluble formate dehydrogenase (S-FDH) is generated in formate induced cells while the membrane-bound formate dehydrogenase (M-FDH) is present under various growth conditions (Cramm, 2009; Oh & Bowien, 1998). However, only S-FDH is required for the growth of *R. eutropha* H16 on formate. The five genes for S-FDH are encoded in one operon on chromosome 1, which is under the control of a σ^{70} promoter that is most likely induced by formate (Oh & Bowien, 1998).

1.2.3 Aerobic and anaerobic growth of *R. eutropha* H16

R. eutropha H16 maintains a very versatile carbon and energy metabolism. The strictly respiratory facultative lithoautotrophic bacterium can grow autotrophically using formate or CO_2 and H_2 as growth substrates and heterotrophically utilizing fructose or numerous organic acids as energy and carbon sources in the presence of oxygen (Cramm, 2009; Pohlmann *et al.*, 2006). Typically, the respiratory chain of *R. eutropha* H16 is composed of a NADH dehydrogenase, a succinate dehydrogenase, a bc_1 complex and three terminal oxidases (Figure 6). The NADH dehydrogenase, also known as complex I, is a protein complex composed of 13 – 14 subunits, which couples electron transfer from NADH to quinones with proton translocation across the membrane (Friedrich *et al.*, 1998; Yagi *et al.*, 1998). The succinate dehydrogenase (complex II), encoded by four genes on chromosome 1, catalyses the oxidation of succinate to fumarate with quinol as electron acceptor. On the other hand, the reduced quinol pool interacts with the quinol-cytochrome c oxidoreductase (bc_1 complex) to oxidize quinol by reducing cytochrome C and translocating protons to the periplasm (Cramm, 2009; Glaeser & Schlegel, 1972). Subsequently, electrons are transported by reduced cytochrome C to the terminal oxidases to catalyse the reduction of O_2 to H_2O and promote coupled proton translocation through the cytoplasmic membrane.

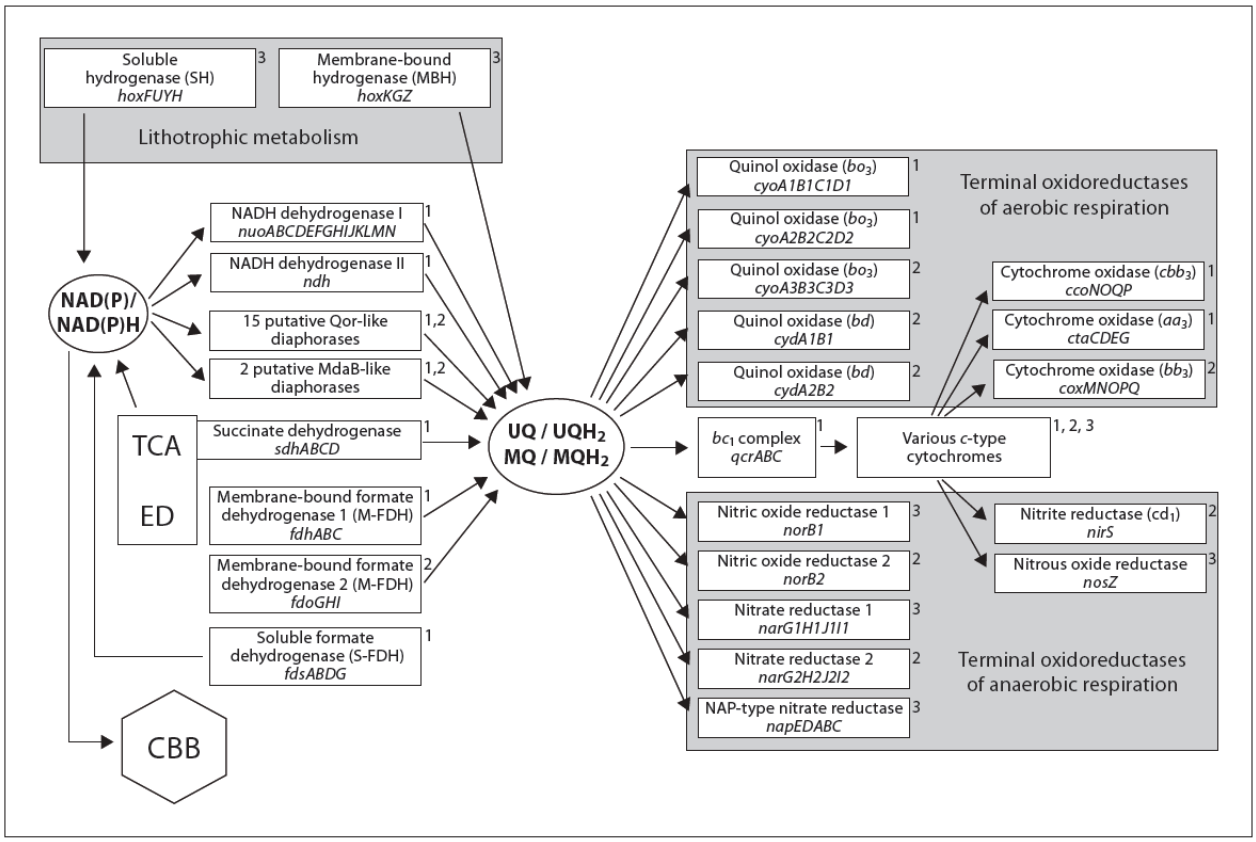


Figure 6: Summary of the energy metabolism of *R. eutropha* H16. Illustrating the main components of the energy metabolism required under autotrophic and heterotrophic growth conditions with oxygen as terminal electron acceptor; or under anaerobic growth conditions with nitrate or nitrite as terminal electron acceptors. Image taken from Cramm (2009).

The genome of *R. eutropha* H16 encodes three genes for cytochrome oxidases and five genes coding for quinol oxidases (Figure 6) allowing for the respiratory chain to adapt to different oxygen concentrations (Cramm, 2009; Pohlmann *et al.*, 2006). In the absence of oxygen the bacterium is able to use nitrate and nitrite as terminal electron acceptors. In total four different terminal oxidoreductases that catalyse the reduction of nitrate (NAR), nitrite (NIR), nitric oxide (NOR) and nitrous oxide (NOS) are encoded on the genome of *R. eutropha* H16. Both, ubiquinone and menaquinone were found to act as electron carriers in *R. eutropha* H16. It is anticipated that aerobic respiration mainly relies on ubiquinone, while menaquinone is predominantly used as an electron carrier during denitrification (Figure 6) (Cramm, 2009).

1.3 Biotechnological application of *R. eutropha* H16

Next to the use for the synthesis of PHAs at large scale, *R. eutropha* H16 has attracted significant interest for a broader range of biotechnological applications including for example the production of proteins and metabolites in high-cell-density fermentation processes. Unlike *E. coli*, *R. eutropha* H16 is able to grow to high-cell-densities under lithoautotrophic and heterotrophic conditions without accumulating growth inhibiting organic acids (Srinivasan *et al.*, 2002). Accordingly, fermentation processes carried out with *R. eutropha* H16 yielded high product concentrations and cell densities of 230 g/l (Ryu *et al.*, 1997; Srinivasan *et al.*, 2002). High-cell-density fermentation processes also offer significant advantages providing improved operating costs, increased productivity and higher product concentrations, enabling substantial improvements of the fermentation processes' economic potential (Andersson *et al.*, 1994; Chen *et al.*, 1992). The synthesis of products based on *R. eutropha* H16 as host included isotope-labelled arginine, 2-methylcitric acid or large amounts of properly folded proteins such as OPH (Ewering *et al.*, 2006; Lutte *et al.*, 2012; Srinivasan *et al.*, 2002). Illustrating the potential of *R. eutropha* H16 as a production host is the ability to form substantial amounts of OPH without significant inclusion body formation in comparison to *E. coli*, which most likely depends on the intracellular redox environment and the bacterium's codon usage (Hess *et al.*, 2015; Srinivasan *et al.*, 2002).

Other applications involving *R. eutropha* H16 aim at utilizing the bacterium's ability to provide sufficient amounts of reducing agents, based on the oxidation of hydrogen as a source of co-factor regeneration. Oxygen-tolerant hydrogenase or transhydrogenase enzymes naturally occurring in *R. eutropha* H16 provide a sufficient basis for co-factor regeneration using molecular hydrogen as substrate, which represents a cheap and clean source for co-factor regeneration that does not produce undesired by-products (Lauterbach *et al.*, 2013; Oda *et al.*, 2013; Pohlmann *et al.*, 2006). This allows the supply of significant amounts of NADH or NADPH for desired reactions without the need to implement heterologous enzyme-coupled approaches for co-factor recycling (Lauterbach *et al.*, 2013; Oda *et al.*, 2013).

In order to implement biotechnological processes and to fully take advantage of the natural capabilities of *R. eutropha* H16, new features need to be established or natural properties refined. A frequently used method for engineering *R. eutropha* H16 aims at the modification of the genomic DNA by homologous recombination. Strains of *R. eutropha* H16 were for example engineered by the integration of T7 polymerase along with several P_{T7} based expression cassettes for the production of OPH or used for the integration of a lactose permease function to enable the use of IPTG-induced

expression systems (Bi *et al.*, 2013; Srinivasan *et al.*, 2002). The same approach was also used to create several knock-out strains for the application of metabolism-based plasmid addiction systems in auxotrophic strains of *R. eutropha* H16 (Budde *et al.*, 2011; Lutte *et al.*, 2012; Voss & Steinbüchel, 2006). However, next to genomic integration, the use of plasmid vectors represents a simple and efficient alternative to introduce new functions.

1.3.1 Design of broad-host-range plasmid vectors

The design of autonomously replicating cloning vectors intended for applications in Gram-negative bacteria relies on the comprehensive study of naturally occurring plasmids or their components. Plasmids are widely spread extrachromosomal, autonomously replicating DNA elements found in eukaryotes, archaea and prokaryotes. A large number of these replicons were identified in plenty of bacterial species encoding a wealth of genetic information and ranging from a few hundred to several hundred thousand basepairs in size (Norman *et al.*, 2009; Tringe *et al.*, 2005). As many plasmids encode features promoting plasmid mobilization and transfer, conjugational plasmids play a crucial role in the exchange of genetic information among different species of bacteria. Along with other mobile genetic elements like transposons or bacteriophages, conjugational plasmids represent an important element of horizontal gene transfer and contribute significantly to the genomic evolution of bacteria (Frost *et al.*, 2005; Smets & Barkay, 2005). A tremendous diversity in genetic information elucidated by horizontal gene transfer shapes prokaryotic genomes and establishes a basis for bacteria to inhabit diverse environmental niches and furthermore promotes microbial ecology (Jain *et al.*, 2003; Koonin & Wolf, 2008; She *et al.*, 2001).

For example, plasmids of the incompatibility group P (IncP) were isolated from different bacteria in freshwater, contaminated soil, pig manure, industrial waste waters and clinical environments (Götz *et al.*, 1996; Pettigrew *et al.*, 1990; Thomas, 1989; Top *et al.*, 1994). This group of self-transmissible, broad-host-range plasmids provides the bacterial host with antibiotic resistance determinants against e.g. kanamycin, penicillin, streptomycin, gentamicin or tetracycline. Other genetically encoded information relates to heavy-metal resistances, multidrug efflux or transporter systems and various operons involved in the degradation of chloroaromatic compounds and environmental toxins (Burlage *et al.*, 1990; Dröge *et al.*, 2000; Thomas, C., Helinski, 1989). Studies performed on the group of IncP plasmids suggest an assignment into three subgroups, namely α, β and γ. The most extensively studied plasmid belongs to the subgroup IncPα and is known as RP4 (RK2) (Pansegrau

et al., 1994; Thomas & Smith, 1987; Thorsted *et al.*, 1998). Like many plasmids of the IncP group, the RP4 plasmid does not only replicate efficiently in a broad range of Gram-negative hosts, but also contains genetic information that promotes mobilization and transfer of the plasmid to other cells by conjugation including mammalian cells (Adamczyk & Jagura-Burdzy, 2003; Thomas, C., Helinski, 1989; Waters, 2001). Plasmid maintenance and stability of RP4 is guaranteed by a site specific recombination unit and a toxin/antidote system (*par* region) (Eberl *et al.*, 1994; Gerlitz *et al.*, 1990; Jiang *et al.*, 2002). Eventually, all of these properties contribute to the efficient propagation and stable maintenance of the RP4 plasmid at a low copy number in a wide range of organisms (Kolatka *et al.*, 2010; Waters, 2001).

The RP4 plasmid, along with plasmids such as RSF1010, p15A, pMB1, pSa or bacteriophages represent an important source for retrieving key genetic elements used for prokaryotic cloning vector design (Ditta *et al.*, 1985; Gruber *et al.*, 2014; Parke, 1990). Such elements include selection markers, promoters, terminators, regulatory elements, mobilization sequences, partitioning sequences and replication elements (Fu, 2006; Pleiss, 2006). Most building blocks and cloning vectors, however, are customized for the use in *E. coli*. Accordingly, the design of plasmid vectors for expression applications in other promising Gram-negative bacterial hosts such as *R. eutropha* H16 requires a different set of features (Kües & Stahl, 1989; Murin *et al.*, 2012; Rangwala *et al.*, 1991; Voss & Steinbüchel, 2006). Comprehensive additional information regarding plasmid vector design intended for the use in *R. eutropha* H16 is provided in chapters 1 to 3.

1.3.2 Plasmid based expression systems for the use in *R. eutropha* H16

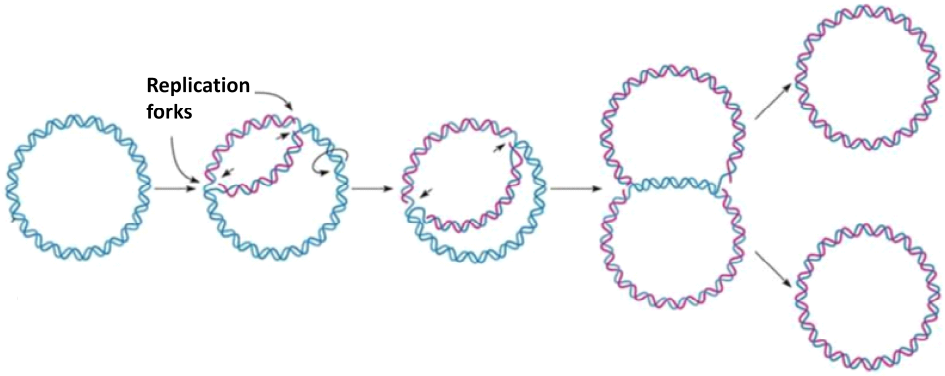
Plasmid replication elements

Numerous expression plasmids were designed for the use in *R. eutropha* H16 on the basis of replication elements derived from broad-host-range plasmids pBBR1, RSF1010, RP4 or the megaplasmid pMOL28 derived from *Ralstonia metallidurans* CH43 (Lutte *et al.*, 2012; Sato *et al.*, 2013; Srinivasan *et al.*, 2003; Voss & Steinbüchel, 2006). These autonomously replicating DNA elements or minireplicons define the plasmid's replication process and substantially influence stable plasmid maintenance as well as the copy number. Plasmid replication processes are usually tightly regulated and require features such as the vegetative origin of replication (*oriV*), replication initiation proteins (Rep proteins) and further elements such as host DNA replication proteins (Kües & Stahl,

1989; del Solar *et al.*, 1998). Essential for plasmid replication is the presence of *oriV* and control element binding sites *in cis*, on the plasmid replicon. If plasmids encode the same replication elements or follow the same replication control mechanism, they cannot be commonly maintained by the same cell and one replicon species will be lost (Kües & Stahl, 1989; del Solar *et al.*, 1998). Accordingly, these replicon species are categorized by incompatibility groups. The replicons used for plasmid design in *R. eutropha* H16 were for example assigned to the following incompatibility groups IncQ (RSF1010) and IncP (RP4), the pBBR1 and pMOL28 replicons are still undefined (Antoine & Locht, 1992; Frey *et al.*, 1992; Pansegrau *et al.*, 1994; Sato *et al.*, 2013). The pBBR1 plasmid was originally isolated from *Bordetella bronchiseptica* and was found to efficiently replicate in a number of Gram-positive and Gram-negative bacteria at a medium copy number. However, the replication mechanism of this broad-host-range plasmid is still unknown (Antoine & Locht, 1992). As in the case of the pBBR1 replicon, the replication mechanism of the pMOL28 has not yet been characterized and expression vectors designed on the basis of pMOL28 replicons are stably maintained at low copy number in *R. eutropha* H16 (Sato *et al.*, 2013).

In general there are three different replication mechanisms known for circular plasmids, the rolling circle mechanism, the strand displacement mechanism and the theta type replication (del Solar *et al.*, 1998). The RP4 replicon for example follows a theta type replication (Kolatka *et al.*, 2010). The replicon contains an AT rich site, DnaA boxes, a replication protein (TrfA) and an *oriV* encoding three iterated motifs necessary for replication (Figure 7) (Kolatka *et al.*, 2010). Replication is initiated by the binding of TrfA and DnaA, a host initiation protein, at the *oriV* to open a replication fork. Subsequently, RP4 plasmid replication occurs in a unidirectional manner in dependence on host factors; generally, the theta type replication can occur unidirectional or bidirectional (Figure 7) (Kolatka *et al.*, 2010; del Solar *et al.*, 1998). The RP4 plasmid and derivatives thereof are able to replicate in a broad range of hosts at a low copy number (Kolatka *et al.*, 2010).

A



B

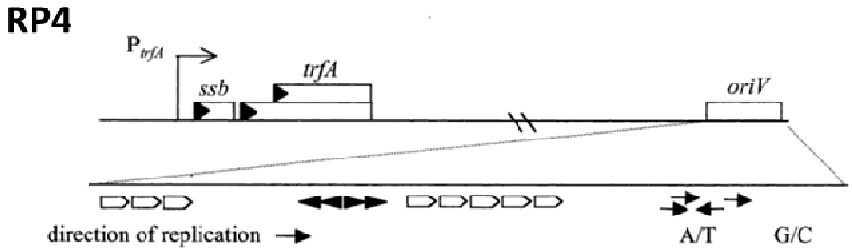


Figure 7: (A) Plasmid replication by the theta type mechanism and (B) elements of the RP4 replicon. (A) Illustration of the theta type replication mechanism, replication forks proceed bidirectional from the *oriV* **(B)** Elements of the RP4 replicon include a *trfA* operon encoding the *trfA* promoter, a short and a long replication initiation protein (TrfA) and a single-strand binding protein (*ssb*). The *oriV* contains iteron sequences (white arrows), AT-rich and GC- rich regions and four DnaA binding boxes (thick black arrow). Image (A) and (B) were adapted from Toukdarian (2004) and Reece & Campbell (2006).

The IncQ plasmids such as RSF1010 replicate by a strand displacement replication mechanism (Frey *et al.*, 1992; del Solar *et al.*, 1998). In this case, three plasmid-encoded proteins are required to initiate replication, a DNA helicase (RepA), a primase (RepB) and the replication initiation protein (RepC). The RSF1010 origin of replication contains one AT and one GC rich region, which are flanked by iteron and inverted repeats sequences (Figure 8) (Frey *et al.*, 1992; del Solar *et al.*, 1998). Replication is initiated by the binding of RepC to the iteron sequence and binding of RepB to the inverted repeats, which enables the formation of a fork-like structure. Subsequently, replication starts from two locations in the *oriV* situated opposite of each other on each DNA strand, namely *ssiA* and *ssiB*. RepB catalyses priming and the DNA strands are synthesised in a bidirectional

manner (Figure 8) (Frey *et al.*, 1992; del Solar *et al.*, 1998). Plasmids of the IncQ family also encode mobilization sequences and an origin of transfer (*oriT*) in the region of the origin of replication. Furthermore, these plasmids exhibit medium copy numbers and are able to replicate in a broad range of hosts including *R. eutropha* H16 (del Solar *et al.*, 1998; Srinivasan *et al.*, 2003).

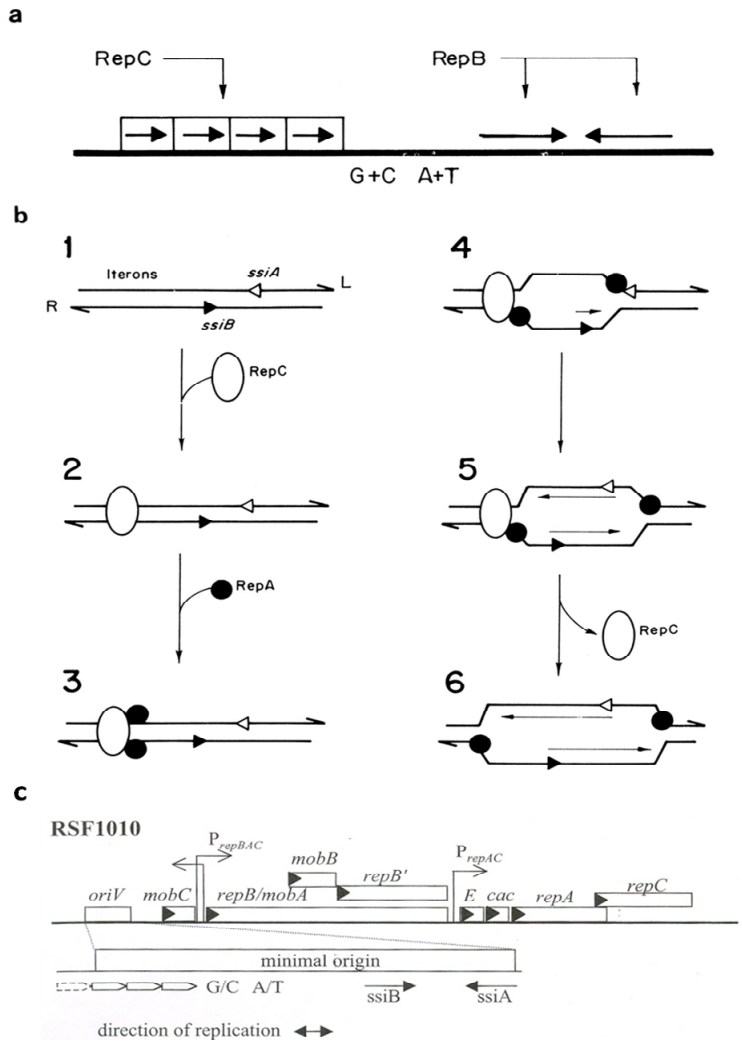


Figure 8: Plasmid replication by the strand displacement mechanism (A) RepB and RepC binding sites (B) the strand displacement replication mechanism (C) RSF1010 origin of replication. (A) Binding of RepC (*oriV* recognition) to interon sequences and RepB (DNA primase) to inverted repeat sequences to initiate plasmid replication. **(B)** Sequential binding of RepC, RepA (DNA helicase) and RepB initiating replication by establishing a replication fork. Replication proceeds bidirectional from *ssiA* and *ssiB* origins **(C)** The RSF1010 origin of replication encodes *mob* genes *mobA*, *mobB*, *mobC*; the origin of conjugational DNA transfer *oriT*; the replication initiation proteins RepA, RepB, RepC; the autoregulatory gene *cac* (control of *repAC*) and the E protein (unknown function); and the *oriV* sequence encoding iteron sequences (white arrows), the single stranded DNA replication initiation regions *ssiA* and *ssiB* as well as GC- and AT-rich regions. Images taken from del Solar *et al.* (1998) and Toukdarian (2004).

In comparison, plasmids replicating by a rolling circle mechanism are processed in a more complex way. In order to start plasmid replication, the plasmid DNA is nicked by the plasmid encoded Rep protein at the *dso*, double stranded origin, exposing the 5'-PO_4 and 3'-OH groups (Figure 9) (del Solar *et al.*, 1998). The free 3'-OH group acts as a starting point for DNA replication and the DNA strand is elongated by host proteins while being displaced at the same time. The replication fork moves along the plasmid DNA in a rolling circle fashion until it reaches the origin of replication (del Solar *et al.*, 1998). The Rep protein then releases the displaced single stranded DNA from the double stranded plasmid, which consists of a parental and the newly synthesised DNA strand. The released single stranded plasmid DNA is complemented in by Lagging-strand synthesis on the basis of the host's RNA and DNA polymerases starting from the single stranded origin, *sso* (Figure 9) (del Solar *et al.*, 1998).

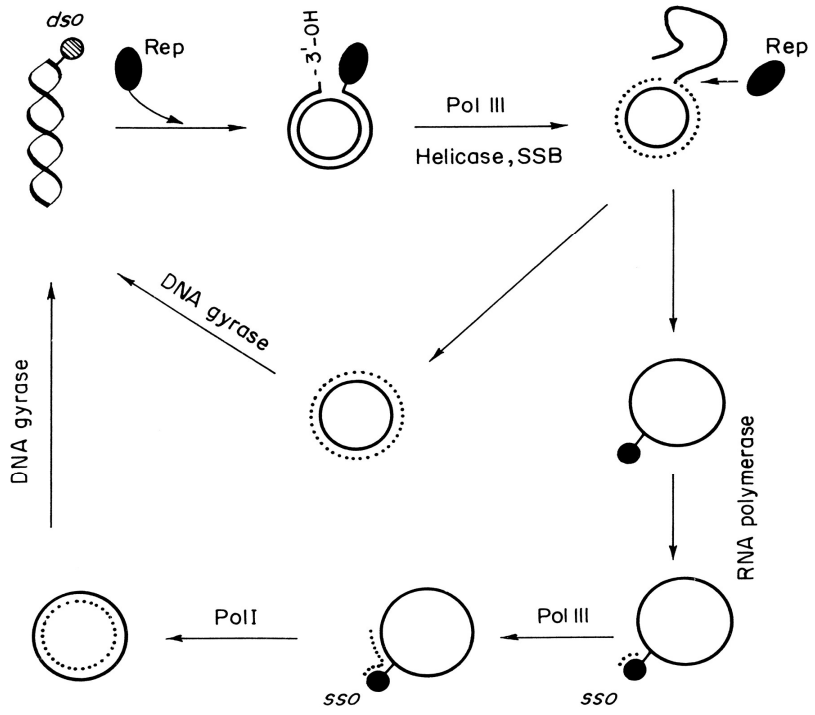


Figure 9: Plasmid replication by the rolling circle mechanism. The plasmid encoded Rep protein binds to the double stranded origin (*dso*) and nicks the DNA at this origin freeing the 3'-OH group. The 5'-PO_4 group remains attached to the Rep protein. Replication starting at the 3'-OH group proceeds in a rolling circle fashion releasing a plasmid consisting of a parental and a newly synthesised DNA strand. Starting from the single stranded origin, *sso*, the remaining single stranded plasmid DNA is complemented in by the host's RNA and DNA polymerases. Image taken from del Solar *et al.* (1998).

Plasmid transfer by conjugation

In general, the efficiency of DNA transformation processes with Gram-negative bacteria is negatively influenced by the complex cell envelope, which causes low transformation efficiences or completely prohibits DNA transfer. In laboratory practice, plasmid transfer on the basis of transformation by physical or chemical methods into *R. eutropha* H16 could only be accomplished with very low yields. However, plasmid DNA transfer to *R. eutropha* H16 was established on the basis of conjugation, a naturally occuring process of DNA transfer that operates with high efficiency (Figure 10) (Steinbüchel *et al.*, 2013).

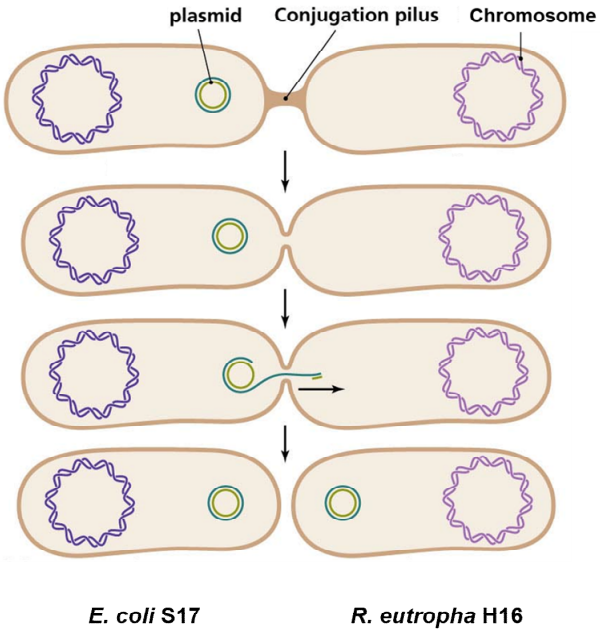


Figure 10: Plasmid conjugation. Plasmid conjugation from *E. coli* S17 to *R. eutropha* H16. Pilus establishes cell-to-cell contact. Afterwards, the plasmid is transferred with the help of conjugative proteins (Dtr and Mob) to *R. eutropha* H16, where the plasmid DNA is established. Image taken from Reece & Campbell (2006).

The process of conjugation requires the presence of several elements inlcuding transfer-genes (*tra*-genes), a mobilization site (*mob*), an origin of transfer (*oriT*), genes coding for the DNA transfer and replication (Dtr) system as well as the mating pair formation (Mpf) system (Daugelavicius *et al.*,

1997; Willetts, 1981). In order for a plasmid to be successfully transferred by the process of conjugation, the *mob* functions including the *oriT* need to be present *in cis*, other functions such as the *tra*-genes can be provided *in trans*. Conjugation of plasmid DNA is based on the formation of a pilus to establish direct cell-to-cell contact (Figure 10) (Waters, 1999; Willetts & Wilkins, 1984). This is accomplished by the proteins of the Mpf system, which form a membrane-spanning protein complex and establish pilus formation. Along with elements of the Dtr system a single-stranded copy of plasmid DNA is passed through the transmembrane pore from the donor to the recipient cell. The single-stranded copy of plasmid DNA is generated by rolling circle DNA replication in the donor cell and reconstituted in the recipient cell after the transfer (Waters, 1999; Willetts & Wilkins, 1984). With *R. eutropha* H16 as the recipient of plasmid DNA, conjugation was accomplished by using *E. coli* S17-1 as donor strain, which facilitates conjugation by chromosomally integrated RP4 *tra*-genes or the use of a helper plasmid providing the *tra*-genes (Simon *et al.*, 1983; Smillie *et al.*, 2010). Conjugation of plasmid DNA from *E. coli* S17-1, carrying the desired plasmid, to *R. eutropha* H16 on the basis of the pBBR1 derived *mob* sequence proved to be efficient (Steinbüchel *et al.*, 2013). However, earlier studies also showed that plasmid transfer by conjugation from *E. coli* to *R. eutropha* H16 can result in substantial deletion events in the plasmid DNA (Schwab *et al.*, 1983).

Stabilization and maintenance of plasmid vectors

All plasmid vectors employed for the use in *R. eutropha* H16 are based on REP, RSF1010, RP4 or pMOL28 minireplicons that replicate efficiently exhibiting low or medium plasmid copy numbers (Lutte *et al.*, 2012; Sato *et al.*, 2013; Srinivasan *et al.*, 2003; Voss & Steinbüchel, 2006). However, despite the use of antibiotic selection recombinant strains of *R. eutropha* H16 carrying these plasmid vectors exhibited significant plasmid loss during fermentation processes, losing at least 90% of plasmid vectors after 70 hours (Lutte *et al.*, 2012; Sato *et al.*, 2013; Srinivasan *et al.*, 2003; Voss & Steinbüchel, 2006). In order to prevent such significant plasmid loss additional elements were implemented to promote plasmid stability and maintenance, including toxin/antidote or metabolism-based addiction systems (Figure 11) (Lutte *et al.*, 2012; Sato *et al.*, 2013; Voss & Steinbüchel, 2006). On the basis of these systems, plasmid stability was significantly improved for all plasmid vectors carrying REP, RP4 or pMOL28 minireplicons (Lutte *et al.*, 2012; Sato *et al.*, 2013; Voss & Steinbüchel, 2006).

Toxin/antidote addiction systems encode a stable toxin, which inhibits growth essential cellular functions, and an unstable antidote that is capable of neutralizing the toxin's activity. The toxin and antidote are propagated along with the cytoplasm during cell division (Figure 11) (Kroll *et al.*, 2010). While the rather stable toxin remains active, the unstable antidote degrades causing growth inhibition or death of plasmid-free cells. If the daughter cells retain a copy of the plasmid and are capable to produce the antidote, the cells remain intact (Kroll *et al.*, 2010; Yamaguchi *et al.*, 2011). The application of a toxin/antidote addiction system on plasmid vectors used in fermentation processes with *R. eutropha* H16 increased plasmid retention rates to 95% over a time period of 96 hours (Sato *et al.*, 2013).

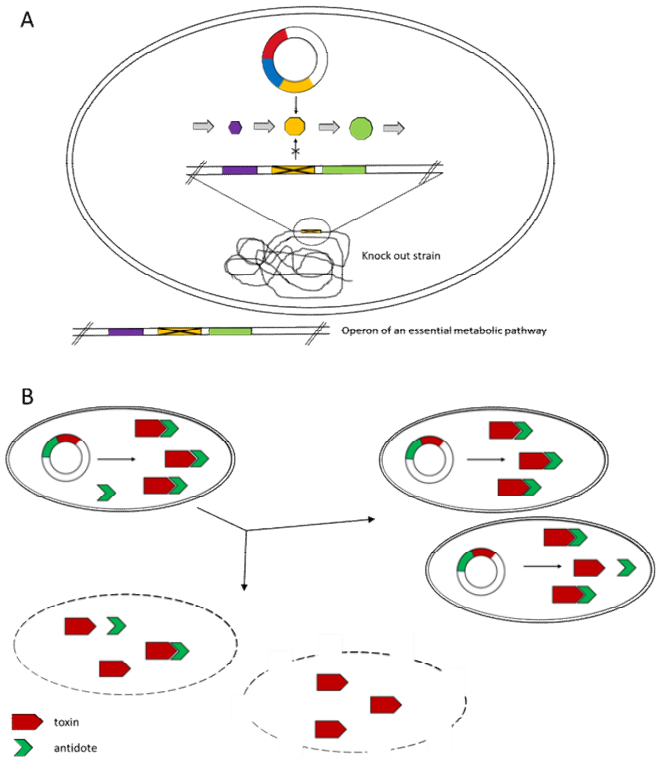


Figure 11: Mechanism of plasmid addiction systems. (A) Metabolism-based plasmid addiction system. Plasmid encoded gene complements essential metabolic function in auxotrophic strains promoting plasmid stability. **(B)** Toxin/antidote plasmid addiction system. Plasmids encode a stable toxin and an unstable antidote. Cells without plasmid are growth inhibited or lyse.

The use of metabolism-based addiction systems, which are based on the complementation of essential metabolic functions in auxotrophic strains of *R. eutropha* H16, did similarly promote significant improvement of plasmid retention rates (Figure 11) (Lutte *et al.*, 2012; Voss & Steinbüchel, 2006). Auxotrophic strains of *R. eutropha* H16 were created by deleting functions such as 2-keto-3-deoxy-6-phospho-gluconate (KDPG) aldolase, which is essential for glycolysis; the hydrogenase transcription factor HoxA, required for lithoautotrophic growth; or pyrroline-5-carboxylate reductase, which is essential for cellular synthesis of proline. As a consequence plasmids carrying the particular gene were stably maintained throughout cultivation enabling growth of auxotrophic *R. eutropha* H16 strains (Budde *et al.*, 2011; Lutte *et al.*, 2012; Voss & Steinbüchel, 2006).

Expression systems

Numerous expression systems have been used to drive or regulate the expression of the gene of interest in *R. eutropha* H16. A number of native promoters derived from pyruvate, PHB, acetoin or *cbb* operons were successfully applied to drive expression along with heterologous promoters such as P_{BAD}, P_{lac}, P_{lacUV5}, P_{tac} and P_{T7} (Barnard *et al.*, 2005; Bi *et al.*, 2013; Delamarre & Batt, 2006; Fukui *et al.*, 2011). Next to the use of constitutive promoters, several inducible expression systems were applied to regulate expression including systems based on the homologous *cbbL* and *phaP* promoters. Gene expression is induced for the P_{cbbL} based system under lithoautotrophic conditions and for the P_{phaP} based inducible expression system by phosphate depletion (Lutte *et al.*, 2012; Srinivasan *et al.*, 2002). In addition a number of heterologous inducible expression systems were applied in *R. eutropha* H16, including regulatory elements like the XylS repressor responding to the inducer m-toluic acid; AraC repressor and the inducer L-arabinose; the TetR repressor responding to the inducer anhydrotetracycline (ATc) or the IPTG-induced expression system based on the LacI repressor and an integrated lactose permease (LacY) function (Bi *et al.*, 2013; Li & Liao, 2015). Additional information regarding the design of plasmid-based expression systems intended for the use in *R. eutropha* H16 is provided in chapters 1 to 3

2. Aim of this study

A focus of this project was to establish a comprehensive toolbox that allows the refinement of natural or the introduction of new features in *R. eutropha* H16 in order to further extend the use of this bacterium as a production host for biotechnological application. Therefore, a set of expression vectors should be designed based on several minireplicons that replicate at different copy numbers and exhibit high rates of plasmid stability. A lack of suitable expression systems applicable in *R. eutropha* H16 requires the identification and characterization of new promoters and regulatory elements facilitating inducible expression under lithoautotrophic or heterotrophic growth conditions. With the design of versatile plasmid vectors the value of *R. eutropha* H16 as production host for metabolites and proteins should further be increased.

Another focus of this project was set on studying the transcription control of the two *cbb* operons encoded in the genome of *R. eutropha* H16. The transcription control of both *cbb* operons in *R. eutropha* H16 is mainly depending on the transcription regulator CbbR, which regulates transcription in response to cellular PEP levels. The CbbR-based regulation represents a feedback control based on the carbon-state of the cell. However, it is expected that *cbb* operon transcription is also influenced by the energy-state of the cell, which still needs to be identified in *R. eutropha* H16.

3. Publications

Chapter 1

3.1 Versatile and stable vectors for efficient gene expression in *Ralstonia eutropha* H16

Contribution to this Chapter

Planning experiments ~ 70%

Laboratory work ~ 85%

Writing manuscript ~ 85%

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Versatile and stable vectors for efficient gene expression in *Ralstonia eutropha* H16

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ABSTRACT

The Gram-negative β-proteobacterium *Ralstonia eutropha* H16 is primarily known for polyhydroxybutyrate (PHB) production and its ability to grow chemolithoautotrophically by using CO_2 and H_2 as sole carbon and energy sources. The majority of metabolic engineering and heterologous expression studies conducted so far rely on a small number of suitable expression systems. Particularly the plasmid based expression systems already developed for the use in *R. eutropha* H16 suffer from high segregational instability and plasmid loss after a short time of fermentation. In order to develop efficient and highly stable plasmid expression vectors for the use in *R. eutropha* H16, a new plasmid design was created including the RP4 partitioning system, as well as various promoters and origins of replication. The application of minireplicons derived from broad-host-range plasmids RSF1010, pBBR1, RP4 and pSa for the construction of expression vectors and the use of numerous, versatile promoters extend the range of feasible expression levels considerably. In particular, the use of promoters derived from the bacteriophage T5 was described for the first time in this work, characterizing the j5 promoter as the strongest promoter yet to be applied in *R. eutropha* I116. Moreover, the implementation of the RP4 partition sequence in plasmid design increased plasmid stability significantly and enables fermentations with marginal plasmid loss of recombinant *R. eutropha* H16 for at least 96 h. The utility of the new vector family in *R. eutropha* H16 is demonstrated by providing expression data with different model proteins and consequently further raises the value of this organism as cell factory for biotechnological applications including protein and metabolite production.

1. Introduction

The facultative chemolithoautotrophic bacterium *Ralstonia eutropha* H16 (now named *Cupriavidus necator* H16) attracted great interest for its diverse metabolic features in recent years. One well known property of the Gram-negative β-proteobacteria is the ability to efficiently produce polyhydroxybutyrate (PHB) in large quantities (Park et al., 2011; Schubert et al., 1988; Steinbüchel, 1998). PHB biosynthesis is triggered under growth limiting conditions and naturally functions as carbon and energy storage in the cells. Intracellular stored PHB can account for as much as 90% of dry cell weight and is already produced on a large industrial scale using *R. eutropha* H16 as host organism (Atlić et al., 2011; Steinbüchel, 1991). Another well studied feature of *R. eutropha* H16 is the ability to grow under chemolithoautrophic conditions. This type of growth is based on autotrophic carbon dioxide fixation accomplished by the enzymes of the Calvin–Benson–Bassham cycle and hydrogen oxidation, which is carried out by [NiFe]-hydrogenases (Bowien and Kusian, 2002; Lenz and Friedrich, 1998). The ability to grow lithoautotrophically enables *R. eutropha* H16 to utilize CO_2 and H_2 as sole carbon and energy sources, respectively. Based on the efficiency of the lithoautotrophic metabolism, the application of this organism for biotechnological strategies of CO_2 utilization has recently attracted great interest (Lütte et al., 2012; Müller et al., 2013).

The main carbon and energy sources utilized by *R. eutropha* H16 under heterotrophic growth conditions include sugars like fructose or *N*-acetylglucosamine and a variety of simple organic acids (Pohlmann et al., 2006). Likewise, hydrogen oxidation can serve as an alternative source of energy due to the O_2 and CO tolerance of the [NiFe]-hydrogenases under various growth conditions (Fritsch et al., 2011). The use of NO_3^- as electron acceptor under anoxic conditions can alternatively be accomplished performing denitrification (Friedrich and Römermann, 1985). Next to the variety of known energy and carbon metabolisms, the metabolic diversity of *R. eutropha* H16 can further be illustrated by the ability to

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0168-1656/

metabolize heavy metals or to degrade a range of chloroaromatic compounds and chemically related pollutants (Pohlmann et al., 2006).

The acquired information about various metabolic features, transcriptome data and the available genome sequence of *R. eutropha* H16 provide a well-established starting point for further development of this organism toward a valuable cell factory by molecular engineering (Brigham et al., 2010, 2012; Peplinski et al., 2010; Pohlmann et al., 2006; Shimizu et al., 2013). Even though several different plasmid based systems were established for the use in *R. eutropha* H16 on basis of autonomous replication or integration into the genome (Lenz et al., 1997; Srinivasan et al., 2002; Voss and Steinbüchel, 2006), there is still a need to develop a comprehensive tool set, which enables the creation of strains expressing homologous and heterologous genes at desired levels. This requires on the one hand, stably maintained expression vectors based on replication systems exhibiting different copy numbers and on the other hand, a choice of suitable promoters that facilitates well-tuned expression of introduced gene functions.

Up to now, a number of prokaryotic expression systems based on well-known promoters were shown to be active in *R. eutropha* H16, namely P_{lac}, P_{tac}, P_{BAD} and P_{T7} (Barnard et al., 2004; Bi et al., 2013; Fukui et al., 2009, 2011). Among these, P_{lac} and P_{tac} act as strong promoters under constitutive expression conditions. However, expression systems based on these promoters cannot be induced due to the inability of *R. eutropha* H16 to take up lactose or isopropyl-β-D-thiogalactopyranoside (IPTG) (Fukui et al., 2011). Other constitutive, native promoters that were used for expression studies in *R. eutropha* H16 derive from operons involved in PHB biosynthesis (P_{phaC}), acetoin metabolism (P_{acoD}, P_{acoX}, P_{acoE}) or the pyruvate metabolism as in case of P_{pdhE}. Nevertheless, all of these native promoters exhibit comparatively weak activity (Delamarre and Batt, 2006). Next to the various, active promoters suitable for the use in *R. eutropha* H16, only a small number of inducible expression systems were characterized up to now. The use of inducible expression systems relies on the L-arabinose inducible BAD promoter, the *phaP* promoter, which is induced by phosphate depletion during PHB synthesis and the *cbbL* promoter that is induced under chemolithoautotrophic conditions (Barnard et al., 2004; Fukui et al., 2002; Lütte et al., 2012). The *phaP* promoter was also used to regulate the bacteriophage derived T7 expression system in *R. eutropha* H16 (Barnard et al., 2004).

A number of autonomously replicating expression vectors were designed for the use in *R. eutropha* H16, based on origins of replication derived from broad-host-range plasmids RP4, RSF1010, pBBR1 and the megaplasmid pMOL28 originating from *Ralstonia metallidurans* CH43 (Ditta et al., 1985; Kovach et al., 1995; Lenz et al., 1997; Sato et al., 2013; Srinivasan et al., 2002). However, significant plasmid loss is reported for all expression vectors relying on these origins of replication during fermentation of *R. eutropha* H16 without applied antibiotic pressure. In case of plasmids carrying a RP4 derived origin of replication, a plasmid loss of 29% was reported after 24 h of fermentation (Lütte et al., 2012). Approximately 90% of RSF1010 based plasmids were lost after 70 h of fermentation and derivatives of plasmids with a pBBR1 origin of replication exhibited a plasmid loss of at least 38% after 24 h of fermentation (Srinivasan et al., 2003; Voss and Steinbüchel, 2006). *R. eutropha* H16 cells carrying expression vectors designed on the basis of pMOL28 origins of replication lost at least 70% of the plasmid after 96 h of fermentation (Sato et al., 2013).

Consequently, several attempts were made to improve plasmid stability for fermentations carried out with *R. eutropha* H16 and significant improvement of plasmid retention could be obtained by including different types of toxin/antitoxin and metabolism-based plasmid addiction systems in plasmid design. The plasmid addiction mechanism of toxin/antitoxin systems relies on two proteins, a toxin and an antitoxin. While the antitoxin is rather unstable, the toxin exhibits high stability and acts on intracellular targets to inhibit cell growth or even cause cell death. A non-toxic toxin/antitoxin complex is formed by both molecules as long as the toxin is expressed along with the antitoxin. However, if the cell looses the plasmid encoding the toxin/antitoxin system, stably retained toxins will kill or inhibit growth of any plasmid-free cell (Kroll et al., 2010; Yamaguchi et al., 2011). Such a toxin/antitoxin system derived from pMOL28, namely *parABS28*, was for instance successfully applied to stabilize expression plasmids in *R. eutropha* H16 over a time period of 96 h with a plasmid retention rate of approximately 100% (Sato et al., 2013).

Besides the use of toxin/antitoxin systems, metabolism-based plasmid addiction systems were also successfully applied in a set of studies to promote plasmid stability in *R. eutropha* H16. In this case, plasmid stability relies on the use of strains that have a metabolic defect and a plasmid that carries the complementing essential gene. One such example is based on *R. eutropha* H16 PHB 4 Δ*eda*, a 2-keto-3-deoxy-6-phospho-gluconate (KDPG) aldolase negative strain unable to utilize carbon sources like fructose or gluconate via the Entner–Doudoroff pathway. In this particular case, a plasmid carrying the KDPG aldolase gene is stably maintained during growth on minimal medium containing fructose or gluconate as sole carbon source resulting in 90% increased plasmid stability compared to plasmid maintenance obtained under antibiotic selection pressure (Voss and Steinbüchel, 2006). In an alternative application, the same defect can be complemented by a plasmid-encoded *xfp* gene encoding a bifunctional xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (Xfp). In this case, the Xfp-dependent pathway presents the only option for viable cell growth of *R. eutropha* H16 PHB 4 Δ*eda* and therefore ensuring stable plasmid maintenance (Fleige et al., 2011). A similar approach was established using the gene encoding the hydrogenase transcription factor HoxA being essential for growth of *R. eutropha* H16 under lithoautotrophic conditions. Plasmids carrying the *hoxA* gene can be stably maintained in the strain *R. eutropha* HF950 lacking *hoxA* under lithoautotrophic growth conditions (Lütte et al., 2012). In another study, a plasmid carrying the essential *proC* gene, coding for pyrroline-5-carboxylate reductase, could be stably maintained in *R. eutropha* H16 Δ*proC* strains (Budde et al., 2011).

The aim of this study was to increase the number of available plasmid based expression systems that are stably maintained in *R. eutropha* H16, in order to further promote the use of this organism as a cell factory for protein and metabolite production; especially in view of employing *R. eutropha* H16 for utilizing CO_2 as carbon source. A set of expression vectors was therefore designed, based on various origins of replication, numerous promoters and the RP4 *par* region. The minireplicons used to construct all expression vectors in this study, were obtained from different broad-host-range plasmids including pSa (IncW), RP4 (IncP), RSF1010 (IncQ) and pBBR1 (incompatibility group not yet defined). The ability to replicate in *R. eutropha* H16 was demonstrated for all of these broad-host-range plasmids in previous studies (Ditta et al., 1985; Kovach et al., 1995; Lenz et al., 1997; Sato et al., 2013; Srinivasan et al., 2002). The RP4 derived partitioning system encoding a site specific recombination system and a toxin/antitoxin system was reported to efficiently stabilize plasmids (Gerlitz et al., 1990; Eberl et al., 1994) and thus was included in the basic plasmid design to increase plasmid propagation and stability. The range of feasible expression levels was obtained by the use of two native promoters and a set of bacteriophage T5 derived promoters that are known to exhibit high activity in *E. coli* cells (Gentz and Bujard, 1985). The bacteriophage T5 derived promoters were hereby characterized as the strongest promoters yet to be applied in *R. eutropha* H16 under constitutive expression conditions.

Table 1
Strains used in this study.

Strain	Description	References or source
E. coli S17-1	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	Invitrogen
E. coli TOP10	F′(*proAB*, *lacIq*, *lacZ*ΔM15, Tn10(tet-r)), *mcrA*, Δ(mrr-hsdRMS-mcrBC), Φ80ΔlacZΔM15, Δ*lacX74*, *deoR*, *recA1*, *araD139*(*ara*, *leu*), 7697, *galU*, *galK*, λ-, *rpsL*(streptomycin-r), *endA1*, *nupG*	Invitrogen
R. eutropha H16	Wild-type, gentamicin resistant	DSMZ428[a]

[a] DSMZ, Deutsche Sammlung für Mikroorganismen und Zellkulturen.

2. Materials and methods

2.1. Strains, plasmids and primers

All strains and plasmids that were used in this study are shown in Tables 1 and 2. Primers used for PCR amplifications are summarized in Table S1 (Supplementary data).

2.2. Cultivation of E. coli and R. eutropha H16 strains

Escherichia coli TOP10 and *E. coli* S17-1 cells were cultivated at 37 °C on lysogeny broth (LB) media with ampicillin [100 μg/ml] or kanamycin [40 μg/ml] according to application. *R. eutropha* H16 cells were cultivated at 28 °C using nutrient broth (NB) or tryptic soy broth (TSB) media supplemented with 0.6% fructose and when needed for selection with gentamicin [20 μg/ml] or kanamycin [200 μg/ml]. All basic media components were purchased from Sigma-Aldrich (St. Louis, MO, USA), Carl Roth (Arlesheim, Germany) and Becton Dickinson and Company (Franklin Lakes, NJ, USA).

2.3. DNA preparation and construction of plasmids

Standard procedures were used for PCR, DNA preparation and manipulation as well as genomic DNA isolation (Sambrook and Russel, 2011). Restriction enzymes, Fast DNA End Repair Kit, Phusion® Polymerase and GeneJET Plasmid Miniprep Kits by Thermo Scientific (Waltham, MA, USA), T4 DNA Ligation reaction mixtures and Wizard® SV Gel and PCR Clean-Up System by Promega (Madison, WI, USA) and Easy-DNA™ Kit by Invitrogen (Carlsbad, California, USA) were used according to the manufacturer's protocols.

2.4. Plasmid construction

The basic plasmid design is illustrated in Fig. 1. All plasmid backbones were constructed on the basis of the pMS470Δ8 plasmid (Fig. S1). The kanamycin resistance gene (Km^r) was amplified with KanR-NotI-fwd, KanR-SpeI-rev primers and introduced in the pMS470 plasmid to exchange Ap^r with Km^r. Subsequently, *egfp* (enhanced green fluorescent protein) and *par* were amplified with RP4-ParA-SpeI-fwd, RP4-ParE-Term-SpeI-rev, EGFP-NdeI-fwd

Table 2
Plasmids used in this work.

Plasmids		
RP4	Broad-host-range plasmid, IncP	Pansegrau et al. (1994)
pSa	Broad-host-range plasmid, IncW	Tait et al. (1982)
pKT230	Broad-host-range expression plasmid, RSF1010 origin of replication, IncQ	Bagdasarian et al. (1981)
pBBR1MCS-5	Broad-host-range expression plasmid, REP origin of replication	Kovach et al. (1995)
pMS470Δ8	Ap^r, P_{tac}	Balzer et al. (1992)
pMS470Ru1	Ap^r, P_{tac}, *estA*	Schwab et al. (2003)
pKRSF1010-P_{tac}-Ru1	Km^r, P_{tac}, *estA*, *par*, *mob*, RSF1010 origin of replication	This work
pKSa-P_{tac}-egfp-mob-pBBR1	Km^r, P_{tac}, *egfp*, *par*, mobilization sequence *mob* from the pBBR1MCS-5 plasmid, pSa origin of replication	This work
pKSa-P_{tac}-egfp-mob-RSF1010	Km^r, P_{tac}, *egfp*, *par*, mobilization sequence *mob* from the RSF1010 plasmid, pSa origin of replication	This work
pKSa-P_{tac}-egfp	Km^r, P_{tac}, *egfp*, partition region *par* from the RP4 plasmid, mobilization sequence *mob* from the RP4 plasmid, pSa origin of replication	This work
pKRep-P_{tac}-egfp	Km^r, P_{tac}, *egfp*, *par*, RP4 *mob*, pBBR1 origin of replication	This work
pKRP4-P_{tac}-egfp	Km^r, P_{tac}, *egfp*, *par*, RP4 *mob*, RP4 origin of replication	This work
pKSaM-P_{tac}-egfp	Km^r, P_{tac}, *egfp*, *par*, RP4 *mob*, pSa origin of replication, contains a mutation in the RepA protein	This work
pKRSF1010-P_{tac}-egfp	Km^r, P_{tac}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{lac}-egfp	Km^r, P_{lac}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{T5}-egfp	Km^r, P_{T5}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{j5}-egfp	Km^r, P_{j5}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{k28a}-egfp	Km^r, P_{k28a}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{k28b}-egfp	Km^r, P_{k28b}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{n25}-egfp	Km^r, P_{n25}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{n26}-egfp	Km^r, P_{n26}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{h22b}-egfp	Km^r, P_{h22b}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{de33}-egfp	Km^r, P_{de33}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{g25}-egfp	Km^r, P_{g25}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{h207}-egfp	Km^r, P_{h207}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{f30}-egfp	Km^r, P_{f30}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-$P_{H16.B1772}$-egfp	Km^r, $P_{H16.B1772}$, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010-P_{groEL}-egfp	Km^r, P_{groEL}, *egfp*, *par*, RSF1010 *mob* and origin of replication	This work
pKRSF1010Δegfp	Km^r, P_{tac}, *par*, RSF1010 *mob* and origin of replication, deleted *egfp*	This work

Km^r, kanamycin resistance; Ap^r,ampicillin resistance; *par*, site specific partitioning system from the RP4 plasmid.

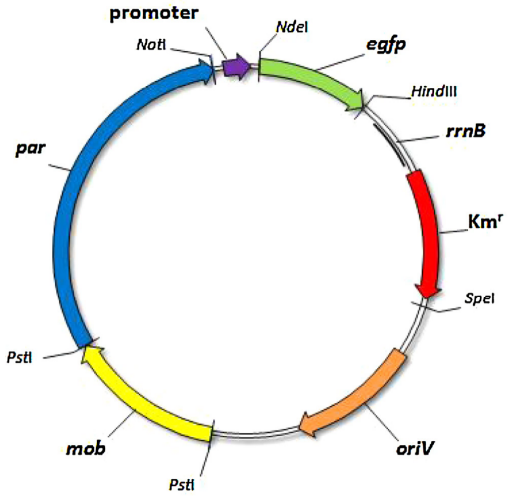

Fig. 1. *Illustration of the basic plasmid design.* The plasmid backbones encode the terminator *rrnB*, RP4 partition region *par*, *egfp*, mobilization sequences *mob* (RP4, RSF1010 or pBBR1), kanamycin resistance Km^r, origin of replication *oriV* (pSaM, pSa, RP4, RSF1010 and REP) and a promoter (P_{groEL}, P_{H16_B1772}, P_{lac}, P_{tac}, P_{h22b}, P_{f30}, P_{de33}, P_{n25}, P_{n26}, P_{g25}, P_{k28a}, P_{T5}, P_{k28b}, P_{h207} and P_{j5}). In case of pKRSF1010 plasmids *mob* and *oriV* are contained in one fragment and introduced via *Spe*I/*Pst*I restriction sites.

and EGFP-HindIII-rev primers; *par* was introduced via *Spe*I/*Pst*I whereas *egfp* was introduced with *Nde*I and *Hind*III restriction sites. In order to create pKRP4-P_{tac}-egfp, pKSa-P_{tac}-egfp, pKSaM-P_{tac}-egfp and pKREP-P_{tac}-egfp the plasmid backbone encoding the tac promoter, *egfp* and *par* was cut with *Spe*I/*Pst*I to introduce the appropriate origin of replication amplified with the following primers: pSa-PstI-fwd, pSa-SpeI-rev, REP-SpeI-fwd, REP-PstI-rev, RP4-oriV1-SpeI-fwd, RP4-oriV1-rev, RP4-oriV2-fwd and RP4-oriV2-PstI-rev. The RP4 derived origin of replication was constructed by fusing the oriV sequence of RP4 to the genes encoding the replication proteins of RP4 using the primers mentioned above. In a second step the RP4 derived mobilization sequence was amplified by RP4-MOB-oriT-PstI-fwd and RP4-MOB-oriT-PstI-rev primers and introduced via *Pst*I/*Pst*I restriction sites into the backbones of pKRP4-P_{tac}-egfp, pKSa-P_{tac}-egfp, pKSaM-P_{tac}-egfp and pKREP-P_{tac}-egfp. The pKRSF1010-P_{tac}-egfp plasmid was constructed by inserting the RSF1010 origin of replication via *Spe*I and *Pst*I restriction sites amplified by RSF1010-SpeI-fwd and RSF1010-PstI-rev primers. Introducing the RP4 mobilization sequence via *Pst*I/*Pst*I was unnecessary, since the RSF1010 origin of replication naturally encodes a complete mobilization sequence. The pKRSF1010-P_{tac}-egfp plasmid was used to insert selected promoters to obtain pKRSF1010-P_{groEL}-egfp, pKRSF1010-P_{H16_B1772}-egfp, pKRSF1010-P_{lac}-egfp, pKRSF1010-P_{h22b}-egfp, pKRSF1010-P_{f30}-egfp, pKRSF1010-P_{de33}-egfp, pKRSF1010-P_{n25}-egfp, pKRSF1010-P_{n26}-egfp, pKRSF1010-P_{g25}-egfp, pKRSF1010-P_{k28a}-egfp, pKRSF1010-P_{T5}-egfp, pKRSF1010-P_{k28b}-egfp, pKRSF1010-P_{h207}-egfp and pKRSF1010-P_{j5}-egfp. H16_B1772, groEL and lac promoters were amplified by Plac-NotI-rev, Plac-BamHI-fwd, PH16_B1772-NotI-fwd, PH16_B1772-NdeI-rev, PgroeL-NotI-fwd and PgroeL-NdeI-rev primers and introduced according to the primer restriction sites. P_{h22b}, P_{f30}, P_{de33}, P_{n25}, P_{n26}, P_{g25}, P_{k28a}, P_{T5}, P_{k28b}, P_{h207} and P_{j5} were amplified using Ph22b-NotI-fwd, Pf30-NotI-fwd, Pde33-NotI-fwd, Pn25-NotI-fwd, Pn26-NotI-fwd, Pg25-NotI-fwd, Pk28a-NotI-fwd, PT5-fwd1, PT5-NotI-fwd2, Pk28b-NotI-fwd, Ph207-NotI-fwd, Pj5-NotI-fwd, and EGFP-HindIII-rev primers and introduced via *Not*I/*Hind*III. P_{groEL} and P_{H16_B1772} are native promoters derived from *R. eutropha* H16. Promoters h22b, f30, de33, n25, n26, g25, k28a, k28b, h207 and j5 derive from bacteriophage T5 (Gentz and Bujard, 1985). Sequences for P_{T5}, P_{tac} and P_{lac} were used as described in Brosius et al. (1985), Ivanov et al. (1990) and Gronenborn (1976). The pKRSF1010Δegfp plasmid was created by cutting pKRSF1010-P_{tac}-egfp with *Nde*I/*Hind*III to remove the insert *(egfp)*, blunted and religated. The pKRSF1010-P_{tac}-Ru1 plasmid was constructed by amplifying *estA* with EstA-NdeI-fwd and EstA-EcoRV-rev primers and introducing *estA* into a pKRSF1010-P_{tac}-egfp backbone cut with *Nde*I/*Hind*III. The *Hind*III restriction site was previously blunted to enable cloning of *estA* via one sticky and one blunt end. *EstA* was amplified from pMS470Ru1 and originates from *Rhodococcus Ruber* (Schwab et al., 2003). Plasmids pKPSa-P_{tac}-egfp-mob-RSF1010 and pKPSa-P_{tac}-egfp-mob-pBBR1 were constructed by inserting mobilization sequences from RSF1010 and pBBR1MCS-5 via *Pst*I/*Pst*I. PBBR1-MOB-PstI-fwd, PBBR1-MOB-PstI-rev, RSF1010-MOB-PstI-fwd and RSF1010-MOB-PstI-rev primers were used to amplify RSF1010 and pBBR1 mobilization sequences.

2.5. Plasmid transfer

E. coli S17-1 cells were transformed with desired plasmids using standard electroporation protocols (Sambrook and Russel, 2011). Plasmid transfer to *R. eutropha* H16 was accomplished by conjugation with *E. coli* S17-1 cells serving as donor strain (Srivastava et al., 1982). The original protocol by Simon et al. (1983) was adapted as follows. Cell suspensions were plated out on TSB gentamicin [20 μg/ml] and kanamycin [200 μg/ml] agar plates for selection of *R. eutropha* H16 transconjugants in the final step.

2.6. Mobilization efficiency

Mobilization sequences derived from pBBR1, RP4 and RSF1010 sequences were evaluated based on their ability to transfer the vector constructs from *E. coli* S17-1 transformants to wildtype *R. eutropha* H16. The mobilization efficiency was determined by the number of colony forming units CFU/ml of *R. eutropha* H16 transconjugants related to the number of CFU/ml of *E. coli* S17-1 transformants before the mobilization (Meyer, 2000).

2.7. Plasmid stability

Cultures of *R. eutropha* H16 strains that contained the desired plasmid were grown over night in liquid TSB media with kanamycin [200 μg/ml]. At a defined starting point 10 ml TSB liquid media were inoculated to an OD_{600} of 0.2 with the overnight culture (ONC) and incubated at 28 °C at 110 rpm for 24 h. After 24 h of incubation this culture was used to inoculate new liquid TSB media of 10 ml to an OD_{600} of 0.2. The new culture was then incubated at 28 °C at 110 rpm for 24 h. This procedure was repeatedly performed at time points 24, 48, 72 and 96 h. Samples of the cultures were taken and dilutions were plated out on TSB agar plates at each time point. All agar plates were incubated for CFU determination at 28 °C for 48 h. In a subsequent step the colonies were transferred from TSB agar plates to TSB kanamycin [200 μg/ml] agar plates by stamping. All agar plates were finally incubated for CFU determination at 28 °C for 48 h. The ratio of CFU that grew on TSB selective and TSB non selective plates at each time point revealed the percentage of plasmid stability over a time period of 96 h. Plasmid stability assays were performed in triplicates.

2.8. Fluorescence unit measurement

Cultures of *R. eutropha* H16 strains were grown in liquid TSB kanamycin [200 μg/ml] media overnight. The ONCs were used to inoculate TSB media to an OD_{600} of 0.2 and grown to a final OD_{600} of approximately 1.5. At this point 200 μl of the culture were used for fluorescence unit measurements with FLUOstar Omega (BMG Labtech, Ortenberg, Germany) at excitation wavelength of 480 nm and emission wavelength of 510 nm, in order to determine eGFP expression levels. Fluorescence units (FU) were determined for *R. eutropha* H16 transconjugants and related to the OD_{600} values of the culture to obtain the relative fluorescence units (RFU). The fluorescence unit measurements over time were performed accordingly. Therefore, samples were drawn and analyzed every 2 h from time point 0 h to 8 h. In either case the RFU values of all samples were related to *R. eutropha* H16 (pKRSF1010Δegfp), which served as negative control.

2.9. SDS-PAGE and Western Blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 4–12% NuPAGE® Bis–Tris Gel (Invitrogen) to separate proteins of the whole cell extract or cell free lysates and pellet fractions. In each case 10 μg of total protein or 0.2 OD_{600} units were added per lane. Transfer of proteins onto a Roti-NC HP40.1, 0.2 μm nitrocellulose membrane (Carl Roth) was accomplished with the TE22 Mini Transfer Tank Unit (Hoefer Inc., Holliston, MA, USA) according to manufacturer's recommendations. A primary mouse (Monoclonal anti GFP, G6795; Sigma-Aldrich) and secondary goat-anti-mouse antibody with a fused horseradish peroxidase (Sigma-Aldrich) were used for eGFP detection on the nitrocellulose membrane. Proteins were visualized using SuperSignal (Pierce, Rockford, USA).

2.9.1. Esterase activity determination and activity stain

SDS-PAGE gels were loaded with lysastes of the EstA expressing strains and afterward incubated in renaturation buffer (699 g/l $NaH_2PO_4 \times 2H_2O$, 2,64 g/l citric acid-monohydrate, pH 6.3) and 30% isopropanol for 45 min. The gels were subsequently washed twice in renaturation buffer for 20 min. In a final step the gels were incubated for 10 min in 10 ml of staining solution (10 ml 0.1 M Tris–HCl, pH 7, 750 μl α-naphthyl acetate (12 mg/ml in acetone) und 250 μl FastBlueB (20 mg/ml)). The presence of EstA was indicated by a purple precipitant. Moreover, cells were disrupted using BugBuster (Merk KGaA, Darmstadt, Germany) to obtain pellet fractions and cell free lysates. The esterase activity assay was carried out photometrically in 0.1 M Tris–HCl buffer, pH 7 containing 4 mM *p*-nitrophenyl butyrate (Sigma-Aldrich) as a substrate. The change in absorption related to *p*-nitrophenol was quantified over time at a wavelength of 405 nm (Schlacher et al., 1998).

3. Results and discussion

3.1. Vector backbone with high mobilization efficiency and high segregational stability

The evaluation and selection of a suitable mobilization sequence was considered in the design of all conjugative plasmids used in this work. The mobilization efficiency was therefore determined for mobilization sequences derived from broad-host-range plasmids RSF1010, RP4 and pBBR1 and is defined as the ability of the mobilization sequence to enable the transfer of a conjugative plasmid from *E. coli* S17-1 transformants to *R. eutropha* H16 (Section 2.6). The mobilization efficiency of the RP4 mobilization sequence was determined to be 7.4×10^{-4} based on 1.5×10^6 CFU/ml of *R. eutropha* H16 (pKPSa-P_{tac}-egfp) transconjugants related to 2.0×10^9 CFU/ml of *E. coli* S17-1 (pKPSa-P_{tac}-egfp) transformants. The RSF1010 mobilization efficiency was determined to be 7.8×10^{-5} based on 1.3×10^5 CFU/ml of *R. eutropha* H16 (pKPSa-P_{tac}-egfp-mob-RSF1010) transconjugants and 1.7×10^9 CFU/ml of *E. coli* S17-1 (pKPSa-P_{tac}-egfp-mob-RSF1010) transformants. Finally, the mobilization efficiency of the pBBR1 mobilization sequence was determined to be 1.4×10^{-8} based on 3.0×10^1 CFU/ml of *R. eutropha* H16 (pKPSa-P_{tac}-egfp-mob-pBBR1) transconjugants related to 2.2×10^9 CFU/ml of *E. coli* S17-1 (pKPSa-P_{tac}-egfp-mob-pBBR1) transformants. The obtained data show that RP4 and RSF1010 mobilization efficiencies were approximately 50,000 times and 5000 times higher compared to the values obtained for the pBBR1 mobilization sequence. Consequently, mobilization sequences derived from RP4 and RSF1010 plasmids were selected as basic elements in expression vector design. The differences in mobilization efficiencies among all mobilization sequences are most likely related to the interaction of transfer and mobilization proteins during the process of conjugation. Since *E. coli* S17-1 cells enable the mobilization of plasmids with the help of a chromosomally integrated RP4 transfer sequence, it is likely that plasmids with mobilization sequences closely related to the natural RP4 transfer and mobilization apparatus interact well and promote high mobilization efficiency.

According to literature, expression vectors designed previously for the use in *R. eutropha* H16 suffer from high segregational instability and plasmids loss after a short time of fermentation (Lütte et al., 2012; Srinivasan et al., 2003; Voss and Steinbüchel, 2006). Consequently, the design of the vector backbone in this work was complemented by including the RP4 derived partitioning system to increase segregational stability and to prevent plasmid loss. Integration of the RP4 partitioning system resulted in 100% plasmid stability of pKPSaM-P_{tac}-egfp, pKRP4-P_{tac}-egfp, pKSa-P_{tac}-egfp, pKRSF1010-P_{tac}-egfp and 95% plasmid stability of pKREP-P_{tac}-egfp after at least 96 h of fermentation in *R. eutropha* H16 (Table 3). In comparison, data reported by several previous studies revealed high rates of plasmid loss during fermentations of plasmid-based recombinant *R. eutropha* H16 strains. Such as a plasmid loss of 29% after 24 h of fermentation for plasmids based on the RP4 origin of replication, approximately 50% for RSF1010 based plasmids and at least 38% of plasmids were lost in case of plasmids based on the pBBR1 origin of replication (Lütte et al., 2012; Srinivasan et al., 2003; Voss and Steinbüchel, 2006). However, significant improvement of plasmid retention could for instance be achieved in case of pBBR1 derived expression vectors relying on auxotrophy and for expression vectors based on the pMOL28 *oriV* by applying the *parABS28* post-segregational killing system from the megaplasmid pMOL28 of *R. metallidurans* CH43 (Budde et al., 2011; Fleige et al., 2011; Lütte et al., 2012; Sato et al., 2013; Voss and Steinbüchel, 2006).

Table 3
Plasmid stability of *R. eutopha* H16 transconjugants over 96 h of fermentation.

Time [h]	pKRP4-P_{tac}-egfp	pKSa-P_{tac}-egfp	pKPSaM-P_{tac}-egfp	pKRSF1010-P_{tac}-egfp	pKREP-P_{tac}-egfp
0	100% ± 0	100% ± 0	100% ± 0	100% ± 0	100% ± 0
24	100% ± 0	100% ± 0	100% ± 0	100% ± 0	98% ± 1.25
48	100% ± 0	100% ± 0	100% ± 0	100% ± 0	98% ± 1.25
72	100% ± 0	100% ± 0	100% ± 0	100% ± 0	97% ± 1.07
96	100% ± 0	100% ± 0	100% ± 0	100% ± 0	95% ± 0.48

In contrast to the plasmid addiction systems mentioned above, the RP4 partitioning system used in this work does not only encode a post-segregational killing system, but furthermore contains a site specific recombination system that supports the resolution of plasmid multimers and is likely to play a crucial role in plasmid propagation to daughter cells (Gerlitz et al., 1990; Eberl et al., 1994; Easter et al., 1998; Sobecky et al., 1996). The post-segregational killing system encoded by *parDE* disables cell growth of plasmid-free daughter cells by inhibiting DNA synthesis. This plasmid addiction system consists of the toxin ParE, which inhibits vital DNA gyrase function and the antitoxin ParD that binds ParE to form a non-toxic toxin/antitoxin complex when expressed. The *parDE* post-segregational killing system exhibits a very similar mode of action as described for *parABS28* from pMOL28 (Jiang et al., 2002; Sato et al., 2013). However, in addition to the encoded post-segregational killing system, the *parCBA* operon codes for three co-translated proteins that contain a nuclease (ParB), a resolvase (ParA) and ParC, a protein of yet unknown function. All *parCBA* encoded proteins act as a plasmid multimer resolution system and are most likely also involved in plasmid propagation to daughter cells (Kristensen et al., 1995). Work done by Eberl et al. (1994) suggests that a plasmid propagation system regulates and assures the distribution of plasmids carrying the RP4 partition region to all daughter cells during cell division.

It could be demonstrated in this work that all *R. eutropha* H16 transconjugants carrying a plasmid with the RP4 partition sequence retained the plasmid at high rates independent of the origin of replication over 96 h of fermentation (Table 3) and proposes a mode of action for this partitioning system essentially independent of the replication system.

3.2. Gene dosage related to different origins of replication in R. eutropha H16

Plasmids pKRSF1010-P_{tac}-egfp, pKRP4-P_{tac}-egfp, pKREP-P_{tac}-egfp, pKPSa-P_{tac}-egfp and pKPSaM-P_{tac}-egfp were compared to each other, in order to enable a characterization based on the gene dosage related to the particular origin of replication in *R. eutropha* H16. All plasmid backbones encoded P_{tac} and *egfp* and only differed in the sequence of the origin of replication (Fig. 1). EGFP expression was determined by measuring the RFU values of *R. eutropha* H16 transconjugants harboring the different plasmids after they were grown to an OD_{600} of approximately 1.5. The highest RFU values were determined for *R. eutropha* H16 (pKREP-P_{tac}-egfp) with 8000 RFU followed by *R. eutropha* H16 (pKRSF1010-P_{tac}-egfp) with 4500 RFU. Lower RFU values were determined for *R. eutropha* H16 (pKPSa-P_{tac}-egfp), (pKRP4-P_{tac}-egfp) and (pKPSaM-P_{tac}-egfp) with RFU values of 2200, 1900 and 900, respectively. The obtained results from the RFU measurements were furthermore confirmed on protein level by SDS-PAGE and Western Blot analysis using a specific eGFP antibody (Fig. 4).

The set of expression vectors used in this work was based on the minireplicons derived from different broad-host-range plasmids, namely RSF1010, RP4, pBBR1 and pSa. The minireplicons of RSF1010, RP4 and pBBR1 were already applied in several studies, but were not compared to each other (Kortlüke et al., 1992; Srinivasan et al., 2003; Voss and Steinbüchel, 2006). Moreover, the pSa derived minireplicon was used for the first time as an *oriV* of an expression vector for the application in *R. eutropha* H16, in this work. A variant of the pSa minireplicon, the pSaM *oriV*, was obtained coincidently by a random single nucleotide mutation that was identified in the replication protein A of the pSaM origin of replication. This resulted in a decrease of RFU values (Figs. 2 and 4) conferring a decrease of the plasmid's copy number. Altogether, the gene dosage of eGFP depending on the origin of replication is illustrated by the broad range of RFU values, from 900 to 8000 RFU (Fig. 2).

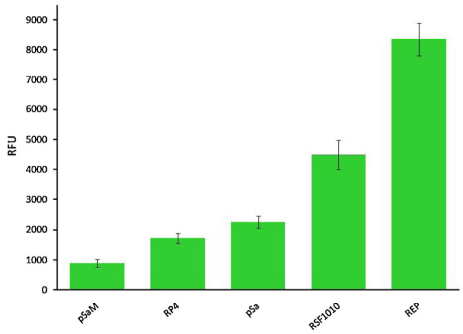

Fig. 2. *Gene dosage of eGFP in R. eutropha H16 based on different origins of replication. R. eutropha* H16 strains containing plasmids pKPSaM-P_{tac}-egfp, pKRP4-P_{tac}-egfp, pKSa-P_{tac}-egfp, pKRSF1010-P_{tac}-egfp and pKREP-P_{tac}-egfp were grown to OD_{600} values of approximately 1.5 and FU values were determined. All FU values were normalized to the particular OD_{600} readings and to the negative control strain *R. eutropha* H16 (pKRSF1010Δegfp) to obtain RFU values.

3.3. Dependence of expression levels on different promoters in R. eutropha H16

The expression of eGFP in *R. eutropha* H16 based on different promoters was determined for P_{groEL}, P_{H16_B1772}, P_{lac}, P_{tac}, P_{h22b}, P_{f30}, P_{de33}, P_{n25}, P_{n26}, P_{g25}, P_{k28a}, P_{T5}, P_{k28b}, P_{h207} and P_{j5}. All plasmids used for this experiment share the same pKRSF1010 backbone and vary only in the promoter sequence driving expression of *egfp*. The j5 promoter was identified as the strongest promoter with 20,000 RFU followed by P_{h207}, P_{k28b} and P_{T5} in the range from approximately 14,000 to 12,000 RFU, P_{k28a} P_{g25}, P_{n26}, P_{n25}, P_{de33} *and* P_{f30} with 11,000 RFU to 9500 RFU, P_{h22b} with 7300 RFU, P_{tac} with 4300 RFU, P_{lac} with 3500 RFU, P_{H16_B1772} with 3100 RFU and P_{groEL} with 600 RFU (Fig. 3). The obtained results from the RFU measurements were furthermore confirmed on protein level by SDS-PAGE and Western Blot analysis using a specific eGFP antibody (Fig. 4).

Up to now, the tac promoter was characterized as the strongest promoter under constitutive expression conditions in *R. eutropha* H16 (Fukui et al., 2011). Consequently, P_{tac} RFU values served as a reference for the other promoters characterized in this work. A large range of expression levels is covered by newly characterized promoters, including the weak groEL promoter, the moderately strong H16_B1772 promoter and the strong T5 phage derived promoters.

The promoters h22b, f30, de33, n25, n26, g25, k28a, T5, k28b, h207 and j5 derived from the bacteriophage T5 were previously described to act as strong promoters in *E. coli* and were now also identified to act as strong promoters in *R. eutropha* H16 (Gentz and Bujard, 1985). Hereby, the j5 promoter proved to be the strongest promoter yet to be applied in *R. eutropha* H16. The range of feasible expression levels in *R. eutropha* H16 was extended considerably based on the selection of various promoters and different origins of replication (Figs. 2, 3 and 4).

In order to determine eGFP expression over time, samples were taken from *R. eutropha* H16 transconjugants containing plasmids pKRSF1010-P_{H16_B1772}-egfp (weak promoter), pKRSF1010-P_{tac}-egfp (medium strength promoter) and pKRSF1010-P_{T5}-egfp (strong promoter) during the exponential growth phase every 2 h. RFU values were determined for all three *R. eutropha* H16 transconjugants and *R. eutropha* H16 (pKRSF1010Δegfp) served as a negative control. The strongest expression level could be determined for

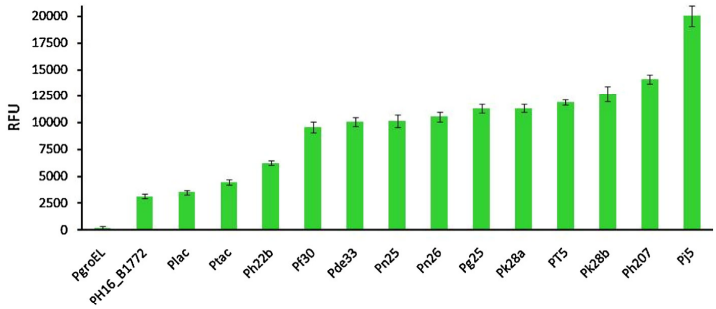

Fig. 3. *Expression levels of eGFP in R. eutropha H16 transconjugants based on various promoters. R. eutropha* H16 strains containing plasmids pKRSF1010-P_{groEL}-egfp, pKRSF1010-P_{H16_B1772}-egfp, pKRSF1010-P_{tac}-egfp, pKRSF1010-P_{tac}-egfp, pKRSF1010-P_{h22b}-egfp, pKRSF1010-P_{f30}-egfp, pKRSF1010-P_{de33}-egfp, pKRSF1010-P_{n25}-egfp, pKRSF1010-P_{n26}-egfp, pKRSF1010-P_{g25}-egfp, pKRSF1010-P_{k28a}-egfp, pKRSF1010-P_{T5}-egfp, pKRSF1010-P_{k28b}-egfp, pKRSF1010-P_{h207}-egfp and pKRSF1010-P_{j5}-egfp were grown to OD_{600} values of approximately 1.5 and RFU values were determined. All FU values were normalized to the particular OD_{600} readings and to the negative control strain *R. eutropha* H16 (pKRSF1010Δegfp) to obtain RFU values.

R. eutropha H16 (pKRSF1010-P_{T5}-egfp), which is approximately 3 times as high as the eGFP expression level of *R. eutropha* H16 (pKRSF1010-P_{tac}-egfp) (Fig. 5). The eGFP expression level of *R. eutropha* H16 (pKRSF1010-P_{H16_B1772}-egfp) was found to be slightly lower compared to *R. eutropha* H16 (pKRSF1010-P_{tac}-egfp). RFU values determined for *R. eutropha* H16 (pKRSF1010-P_{H16_B1772}-egfp) rise from 40 RFU at 0 h to 3100 RFU after 8 h of fermentation. The growth pattern during the exponential growth phase showed that *R. eutropha* H16 (pKRSF1010-P_{T5}-egfp) has the slowest growth rate followed by *R. eutropha* H16 (pKRSF1010-P_{tac}-egfp) and (pKRSF1010-P_{H16_B1772}-egfp). The non-expressing control strain *R. eutropha* H16 (pKRSF1010Δegfp) exhibited the fastest growth pattern. As expected the growth rate of *R. eutropha* H16 transconjugants decreases with the strength of constitutive eGFP expression.

In addition to eGFP, which is widely used as a reporter protein, the esterase EstA originating from *Rhodococcus ruber* was chosen to evaluate the protein expression properties in *R. eutropha* H16 (Schwab et al., 2003). Therefore the expression of the esterase EstA protein was monitored by SDS-PAGE (Fig. 6) and the activity of the enzyme was determined toward two different substrates (*p*-nitrophenyl butyrate and α-naphthyl acetate) with the strain *R. eutropha* H16 carrying the plasmid pKRSF1010-P_{tac}-Ru1 and for comparison with the strains *E. coli* BL21 (pKRSF1010-P_{tac}-Ru1) and *E. coli* BL21 (pMS470Ru1). The plasmid pMS470Ru1 is an inducible high copy plasmid which contains the tac promoter and exhibits the highest expression levels of EstA reported in

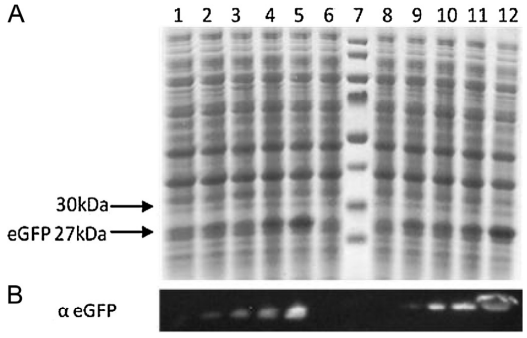

Fig. 4. *SDS-PAGE and Western Blot illustrating eGFP expression in R. eutropha H16 based on different promoters and origins of replication.* (A) SDS-PAGE of whole cell lysates of *R. eutropha* H16 transconjugants carrying the following plasmids: lane 1: pPSaM-P_{tac}-egfp, lane 2: pRP4-P_{tac}-egfp, lane 3: pPSa-P_{tac}-egfp, lane 4: pRSF1010-P_{tac}-egfp, lane 5: pREP-P_{tac}-egfp, lane 6: pKRSF1010Δegfp. Lane 7: PageRuler Prestained Protein Standard (Fermentas), lane 8: pKRSF1010-P_{groEL}-egfp, lane 9: pKRSF1010-P_{H16_B1772}-egfp, lane 10: pKRSF1010-P_{lac}-egfp, lane 11: pKRSF1010-P_{tac}-egfp and lane 12: pKRSF1010-P_{T5}-egfp. (B) Western Blot of whole cell lysates of *R. eutropha* H16 transconjugants. EGFP was detected with a monoclonal GFP antibody (α-eGFP); lane 1: pPSaM-P_{tac}-egfp, lane 2: pRP4-P_{tac}-egfp, lane 3: pPSa-P_{tac}-egfp, lane 4: pRSF1010-P_{tac}-egfp, lane 5: pREP-P_{tac}-egfp, lane 6: pKRSF1010Δegfp. Lane 7: PageRuler Prestained Protein Standard (Fermentas), lane 8: pKRSF1010-P_{groEL}-egfp, lane 9: pKRSF1010-P_{H16_B1772}-egfp, lane 10: pKRSF1010-P_{lac}-egfp, lane 11: pKRSF1010-P_{tac}-egfp and lane 12: pKRSF1010-P_{T5}-egfp. Total protein of 0.2 OD_{600} units was added per lane.

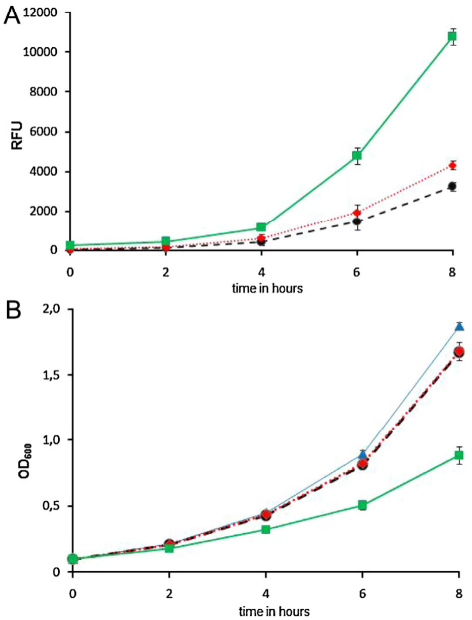

Fig. 5. *Time course of eGFP expression (A) and growth (B) of R. eutropha H16 containing plasmids. R. eutropha* H16 transconjugants harboring plasmids pKRSF1010-P_{H16_B1772}-egfp (black, dashed line), pKRSF1010-P_{tac}-egfp (red, dotted line) and pKRSF1010-P_{T5}-egfp (green, thick line). The blue thin line relates to the control *R. eutropha* H16 (pKRSF1010Δegfp). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

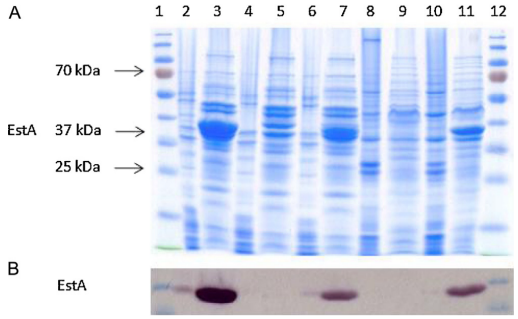

Fig. 6. *Coomassie and activity stained SDS-PAGE illustrating R. ruber EstA expression in different E. coli BL21 and R. eutropha H16 strains.* (A) SDS-PAGE: lane 1: PageRuler Prestained Protein Standard (Fermentas), lane 2: *E. coli* BL21 (pMS470Ru1) pellet fraction, lane 3: *E. coli* BL21 (pMS470Ru1) cell free lysate, lane 4: *E. coli* BL21 pellet fraction, lane 5: *E. coli* BL21 cell free lysate, lane 6: *E. coli* BL21 (pKRSF1010-P_{tac}-Ru1) pellet fraction, lane 7: *E. coli* BL21 (pKRSF1010-P_{tac}-Ru1) cell free lysate, lane 8: *R. eutropha* H16 pellet fraction, lane 9: *R. eutropha* H16 cell free lysate, lane 10: *R. eutropha* H16 (pKRSF1010-P_{tac}-Ru1) pellet fraction, lane 11: *R. eutropha* H16 (pKRSF1010-P_{tac}-Ru1) pellet fraction, lane 12: PageRuler Prestained Protein Standard (Fermentas). (B) Activity stain: SDS-PAGE incubated in renaturation buffer and stained EstA bands based on α-naphthyl acetate and FastBlueB. Samples loaded in the same order. An equivalent of 0.2 OD_{600} units of the pellet fractions and 10 μg of cell free lysates were added per lane.

E. coli BL21 (Schwab et al., 2003). In contrast to that the plasmid pKRSF1010-P_{tac}-Ru1 was described as a low copy plasmid earlier in this work. In all these strains the esterase EstA was expressed by the tac promoter, in the case of pKRSF1010-P_{tac}-Ru1 under constitutive and with pMS470Ru1 under inducible expression conditions. As can be seen from Fig. 6, high amounts of active protein are expressed after induction and overnight fermentation with *E. coli* BL21 (pMS470Ru1). Constitutive expression of the esterase EstA driven by the tac promoter did produce similar amounts of protein in the cell free lysate of *E. coli* BL21 and *R. eutropha* H16 (Fig. 6). The volumetric activity values for EstA related to the protein content of the cell free lysate resulted in specific activity values of 30.1 U/mg for *E. coli* BL21 (pMS470Ru1), 5 U/mg for *E. coli* BL21 (pKRSF1010-P_{tac}-Ru1) and 4 U/mg for *R. eutropha* H16 (pKRSF1010-P_{tac}-Ru1). The obtained data furthermore indicate that *R. eutropha* H16 is able to produce active EstA under constitutive expression conditions in comparable amounts to *E. coli* BL21.

In summary, these results clearly demonstrate that *R. eutropha* H16 is a promising host for protein expression. Current work focuses on establishing regulated expression systems based on the promoters described in this work which should further raise the value of this organism as cell factory for biotechnological applications including protein and metabolite production.

4. Conclusion

With this study we could demonstrate that the chemolithoautotrophic bacterium *R. eutropha* H16 is a valuable expression host for protein production. One major problem encountered so far was the high instability of expression vectors in this organism. This could be efficiently overcome by including the partitioning region of the plasmid RP4 into the expression vectors. All applied replicons were efficiently stabilized even at high burden on cells caused by constitutive expression of proteins. The range of feasible expression levels in *R. eutropha* H16 was moreover significantly increased by the use of promoters derived from bacteriophage T5, which exhibit strong expression in *E. coli* as well as in *R. eutropha* H16 cells. The bacteriophage T5 derived promoters were hereby characterized as the strongest promoters yet to be applied in *R. eutropha* H16 under constitutive expression conditions.

The high potential of this organism to produce properly folded proteins, even under significant stress conditions provided by constitutive expression from strong promoters, was demonstrated by using an esterase originating from a bacterium with a higher GC content as model protein for functional expression. To summarize, this study increases the number of versatile and stable plasmid based expression systems for the use in *R. eutropha* H16, which further promotes the application of this organism as a cell factory for protein and metabolite production.

Authors' contributions

SG, PK, HS planned and started the project. Wet laboratory work was carried out by SG, JH, PK. SG and PK wrote the manuscript. All authors read, corrected and approved the final version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.06.030.

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Chapter 2

3.2 Versatile plasmid-based expression systems for Gram-negative bacteria – General essentials exemplified with the bacterium *Ralstonia eutropha* H16

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REVIEW

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Review

Versatile plasmid-based expression systems for Gram-negative bacteria—General essentials exemplified with the bacterium *Ralstonia eutropha* H16

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Abstract

The Gram-negative bacterium *Escherichia coli* is currently the most efficient and widely used prokaryotic host for recombinant protein and metabolite production. However, due to some limitations and to various interesting features of other Gram-negative bacteria efficient vector systems applicable to a broad range are desired. Basic building blocks for plasmid-based vectors include besides the need for a suitable selection marker in the first line a proper replication and maintenance system. In addition to these basic requirements, further elements are needed for Gram-negative bacteria beyond *E. coli*, such as *Pseudomonas pudita, Ralstonia eutropha, Burkholderia glumae or Acinetobacter* sp.. Established building blocks have to be adapted and new building blocks providing the desired functions need to be identified and exploited. This minireview addresses so far described and used genetic elements for broad host range replication, efficient plasmid maintenance, and conjugative plasmid transfer as well as expression elements and protein secretion signals. The industrially important bacterium *R. eutropha* H16 was chosen as a model organism to provide specific data on the effectivity and utility of building blocks based on such genetic elements.

Contents

Introduction

Along with other sources, such as bacterial and bacteriophage genomes, naturally occurring plasmids represent an important source of genetic elements that can be exploited for vector design and setup. This includes in addition to basic maintenance function components selection markers, promoters, terminators and regulatory elements for expression. Altogether, a large variety of cloning vectors and their elements were characterized and

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optimized for the use in bacterial expression hosts [1,2]. However, the majority of these vectors were customized for the application in *Escherichia coli*; the prokaryotic organism of choice for recombinant protein production [3–5]. Even though the *E. coli* system is highly developed, due to the manifold metabolic capabilities and diverse protein reservoir, there exists a strong need for developing vector systems applicable to a broad range of Gram-negative bacteria as they exhibit a large spectrum of metabolic activities that provide intermediates, end products and pathways with potential application in biotechnology. Moreover, various expression hosts next to *E. coli* still offer advantages related to protein stability and metabolite production [6]. *Ralstonia eutropha* H16 for example is capable to heterologously express active and soluble organophosphohydrolase (OPH); contrarily expression of this protein in *E. coli* results in mainly insoluble and inactive product [7]. Furthermore, the production of specific alkaline lipases and biosurfactants on industrial level is accomplished by the use of *Acinetobacter* sp. [8]. Yet another example represents the use of *Pseudomonas fluorescens* as an expression platform for large scale fermentation processes. The application of this *Pseudomonas* strain is beneficial for particular processes due to the absence of significant acetate accumulation during cultivation, which can be a constraining factor for *E. coli* based fermentations [9].

In order to access a broad range of promising Gram-negative bacterial hosts, such as *Pseudomonas pudita*, *P. fluorescens*, *R. eutropha*, *Burkholderia glumae* or *Acinetobacter* sp., the design of vectors for expression applications requires an adapted set of features [6,10–12]. This finds a good basis with the existing comprehensive knowledge of naturally occurring plasmids or their components. A large number of plasmids were identified in plenty of bacterial species encoding a wealth of genetic information and ranging from a few hundred to several hundred thousand basepairs in size [13,14]. One highly interesting group of plasmids that is well-studied and also used to obtain various elements for cloning vector design is belonging to the incompatibility group P-1 [15–17]. Isolated from different bacteria in freshwater, contaminated soil, pig manure, industrial waste waters and clinical environments [18–21], this group of self-transmissible plasmids replicates efficiently in a broad range of Gram-negative hosts [22–24]. Among these the IncP-1α plasmid RP4 (RK2) is one of the most extensively studied plasmids [25–27]. It contains several genetic elements contributing to the efficient propagation and stable maintenance at a low copy number in a wide range of organisms [24,28–30]. Widespread application of key genetic elements for cloning vector design from the RP4 plasmid include a site specific recombination unit (*parCBA*) and a toxin/antidote system (*parDE*) that mediate stable maintenance of the RP4 plasmid resulting in high segregational stability, the replication elements including the origin of replication (*oriV*) and *trans*-acting replication functions (*trfA*, *trfB*) as well as the mobilization function (*mob*) [31–34]. Accordingly, a variety of building blocks that are functionally active in a broad range of Gram-negative hosts, like replication elements, resistance markers, mobilization sequences or promoter systems, were obtained from genomes of bacteria, bacteriophages or other naturally occurring plasmids such as pBBR1, RSF1010, p15A, pMB1 or pSa. This includes for example well-characterized elements like the T7 bacteriophage RNA-polymerase/promoter expression system, bacteriophage T5 derived promoters, the *E. coli lac* promoter, RSF1010 or pBBR1 derived replication elements and the lacO/LacI or the araO/AraC regulatory systems [35–39]. Overall the pool of building blocks is continuously processed and extended by genetic engineering of existing building blocks or the design of entirely new features. Representatives of this group of building blocks are elements like the *tac* promoter, rationally designed bifunctional promoters or the rtTA2^S-M2 (Tet-on) transactivator [40–42]. Altogether, comprehensive characterization and skilful combination of functionally active genetic elements create a basis for metabolic engineering and protein production in the host of choice.

This review discusses the significance of several building blocks such as replication elements, promoter systems, partitioning sequences, selection markers and mobilization sequences in regard to the design of stably maintained and versatile broad-host-range expression vectors. Hereby, the β-proteobacterium *R. eutropha* H16 will serve as a representative Gram-negative expression host of choice that is already used for plasmid based protein and metabolite production.

The model organism *Ralstonia eutropha* H16

R. eutropha H16, recently reclassified as *Cupriavidus necator* H16, is a facultative chemolithoautotrophic, Gram-negative soil bacterium. Like many other β-proteobacteria, it carries a multi replicon genome, which is comprised of two chromosomes and one megaplasmid encoding highly versatile metabolic features such as the ability to produce polyhydroxybutyrate (PHB), to degrade chloroaromatic and chemically related pollutants or to perform denitrification [43–45]. An existing and compelling flexibility of the bioenergetic metabolism can for instance be attributed to its ability to grow lithoautotrophically, enabling the organism to metabolize CO_2 and H_2 as sole carbon and energy sources in the absence of organic growth substrates [46–48]. Heterotrophic growth on the contrary, depends on sugars like fructose or *N*-acetylglucosamin and a variety of simple organic acids as main carbon and energy sources [43,49]. Furthermore, denitrification is performed under anaerobic conditions by using nitrate as an alternative electron acceptor [50]. These metabolic key features promoted *R. eutropha* H16 as a model organism for the study of mechanisms involved in the control of CO_2 fixation, hydrogen oxidation and denitrification.

Another area of high technological interest is the PHB biosynthesis capability of *R. eutropha* H16, for which the organism still receives significant attention [43,45,51,52]. Nonetheless, next to these main areas of focus, *R. eutropha* H16 attained attraction as an expression host for the production of metabolites and proteins under lithoautotrophic as well as heterotrophic growth conditions [53–55]. Supplementary studies demonstrated the feasibility of high cell density fermentations of up to 230 g/L biomass and the lack of detectable inclusion body formation, indicating promising key features for further improvement of the wild-type *R. eutropha* H16 strain as an alternative expression host [56,57].

Up to now, several plasmid based expression systems were developed for the use in *R. eutropha* H16 applying different approaches and employing a variety of building blocks including replication elements, plasmid stability, segregation and expression related features [12,56,58–60]. Hereby, the choice of replication

elements is crucial, since these significantly affect host compatibility, copy number and plasmid stability.

Vector building blocks

Replication elements

Plasmid based expression systems that have been constructed for many Gram-negative bacteria including *R. eutropha* H16 rely on autonomously replicating DNA elements (minireplicons), defining their replication capabilities. A properly regulated replication system plays a crucial role for stable maintenance and defined copy number of the expression vector. The particular copy number of such minireplicons is defined by plasmid-encoded elements regulating and tightly controlling the replication process [61,62]. Regions and loci essential for replication are: (i) the vegetative origin of replication (*oriV*), (ii) a replication initiation protein (Rep proteins) that binds to cognate sites in the *oriV* region, and (iii) elements involved in the control of replication [61]. Whereas *oriV* and binding sites for control elements have to be present *in cis* on the plasmid replicon, the other functions can be supplied *in trans* by host elements. In general, three different mechanisms are known for the replication of circular plasmids: the theta type, the strand displacement and the rolling circle replication. Plasmids employing a theta-replicating or strand displacement mechanism seem to occur more often in Gram-negative bacteria, whereas plasmids replicating on the basis of the rolling circle mechanism are mainly found in Gram-positive bacteria [11,61]. Progressing studies revealed that plasmids applying the same replication control system cannot be maintained in one cell at the same time, resulting in the loss of one replicon species. Therefore, plasmids are categorized by their mode of replication control and assigned to incompatibility groups. Various well-characterized broad-host-range plasmids isolated from Gram-negative bacteria can for instance be allocated to incompatibility groups IncP (e.g. RP4), IncQ (e.g. RSF1010), IncN (e.g. pCU1), IncW (e.g. pSA) and IncA/C (e.g. RA1) [63–65].

A frequently used origin of replication for constructing broad-host-range expression vectors is obtained from the 60 kbp RP4 plasmid (also known as RK2), a theta-replicating plasmid of the IncP-1α incompatibility group [25,34,66]. RP4 replicates and is stably maintained in a great variety of Gram-negative bacteria. The regulation of the plasmid's copy number is accomplished by the interaction of the TrfA protein with the *oriV* in the so called handcuffing process [67–69]. A minimal DNA sequence of 617 bp of the RP4 plasmid was identified to fully act as functional *oriV* [70]. An even smaller 393 bp subfragment thereof could be used to successfully initiate replication in some hosts such as *E. coli* and, less efficiently, in *Pseudomonas putida* [71]. However, only the 617 bp long *oriV* sequence is able to efficiently mediate replication in *R. eutropha* H16 and was furthermore used for the construction of several expression vectors [58,34,72].

Plasmids belonging to the IncQ group are relatively small in size, R300b with 9 kbp or RSF1010 with 8.7 kbp for example, and exhibit moderate copy numbers [73,11,74]. All plasmids of the IncQ group encode an initiation protein (RepC), a helicase (RepA) and a primase (RepB), which enable autonomous replication and independence from the host's replication apparatus [75,76]. The basic structure of the RSF1010 replicon contains two *trans*-acting regions and one *cis*-acting region, which are separated by mobilization genes and an origin of transfer (*oriT*). The *cis*-region containing the *oriV* regulates replication and determines incompatibility [74,77]. Structurally this region is similar to regions found in the *oriV* of the RP4 plasmid [61,78,79]. The three proteins RepA, RepB, RepC that are necessary for replication initiation and plasmid copy-number control are encoded in the two *trans*-regions of the RSF1010 basic replicon [76]. The RSF1010 plasmid itself and expression vectors based on RSF1010 minireplicons are successfully replicating in a very broad range of hosts including *R. eutropha* H16 [56,58,64,80,81].

As in the case of the RP4 and RSF1010 plasmids, the large sized, broad-host-range plasmids of the IncW family are found in a variety of different bacterial species [82]. The most famous representative of the IncW group is the pSa plasmid, isolated from *Shigella* sp. [83]. The replication process of pSa and other IncW plasmids was found to proceed bidirectionally from the *oriV*, in the fashion of a theta replication mechanism. The pSa plasmid exhibits a low copy number of two to three copies per cell [63]. Regions essential for the replication process include an *oriV* as well as an operon containing two ORFs coding for the resolvase ResP and RepA, a replication initiator protein [84]. Replication of IncW plasmids was observed in numerous different bacteria, hereby the pSa plasmid was identified to replicate in *R. eutropha* H16. It could recently also be demonstrated that expression vectors on the basis of a pSa minireplicon are functionally replicating in *R. eutropha* H16 at a low copy number [58].

An additional noteworthy broad-host-range plasmid is pBBR1. This plasmid was originally isolated from *Bordetella bronchiseptica* and replicates in a variety of Gram-negative bacteria [85]. The molecular basis of the pBBR1 replication is still not understood and may represent a new incompatibility group [86]. Nevertheless, a replication protein RepA sharing sequence homologies with other proteins involved in replication initiation, is encoded in the putative region responsible for vegetative replication of the pBBR1 plasmid [85]. The plasmid was estimated to have a medium copy number of up to 10 copies per cell in *Bordetella* species. Since the replicon is stably maintained in several Gram-negative bacteria, pBBR1 is frequently used for the construction of broad-host-range expression vectors such as the widely used pBBR1MCS vector series and its derivatives [87–90]. A successful application of pBBR1 based expression vectors was also reported for *R. eutropha* H16 [12,58].

Furthermore, other replication elements originating from large megaplasmids, like pMOL28 of *Ralstonia metallidurans* CH43, were used for the construction of expression vectors and could also be successfully applied in *R. eutropha* H16 [60].

Plasmid stability and maintenance strategies

Overall the design of expression vectors that are suitable for the use in a wide range of Gram-negative bacteria needs to be reassessed with respect to requirements concerning the host bacteria and the kind of application. Even though pMOL28, pBBR1, RP4, RSF1010 and pSa minireplicons were applied successfully with expression vectors for the production of proteins and metabolites in *R. eutropha* H16 (Table 1), a significant loss of plasmids was observed in any case despite the use of antibiotic selection [12,54,57,60]. In consequence, mechanisms providing high segregational stability need to be implemented in the design of these broad-host-range expression vectors.

TABLE 1

Overview of vector building blocks

Replication elements		
Building block	**Property**	**Source**
pMOL28	Incompatibility undefined, low copy number replicon	[60]
RP4	IncP, low copy number replicon	[25]
pSa	IncW, low copy number replicon	[63]
RSF1010	IncQ, medium copy number replicon	[64]
pBBR1	Incompatibility undefined, medium copy number replicon	[85]
Plasmid stability and maintenance		
Building block	Property	Source
Essential metabolic gene	Complements a knock-out strain (auxotrophy)	[91]
pMOL28 *parABS28*	Toxin/antidote system	[60]
RP4 *parDE*	Toxin/antidote system	[32]
RP4 *parCBA*	Multimer resolution and partitioning system	[31]
Mobilization and transfer sequences		
Building block	Property	Source
RP4 transfer genes	Essential to promote plasmid transfer	[25]
RP4 mob sequence	Very effective plasmid mobilization in combination with RP4 transfer genes	[25]
RSF1010 mob sequence	Effective plasmid mobilization in combination with RP4 transfer genes	[64]
pBBR1 mob sequence	Poor plasmid mobilization in combination with RP4 transfer genes	[85]

In general stable maintenance of plasmids in bacterial cells is usually achieved when the genetic information encoded on the plasmid is advantageous for the host. Various antibiotic or heavy-metal resistances encoded on plasmids can represent a valuable selective advantage for the host bacterium under particular growth conditions and therefore promote the maintenance of the plasmid by the host [62,69,91]. In genetic engineering approaches antibiotic selection markers are frequently used to accomplish the maintenance of expression vectors in bacterial cultures. However, the use of antibiotics in large scale fermentations is not favoured due to significant associated financial expenses and negative environmental impact [3,92]. In addition, plasmid stability assays revealed that an increasing number of cells tend to lose the plasmid during fermentation despite the use of antibiotic pressure. This could for example also be observed and quantified for fermentations carried out with recombinant *R. eutropha* H16 strains. Plasmid loss of up to 90% after 70 h of fermentation could be observed with various expression vector constructs despite the use of antibiotic selection [12,54,57,60].

Alternatively, other naturally occurring mechanisms promoting plasmid stability and maintenance can be used for the design of expression vectors including toxin/antitdote addiction systems, plasmid multimer resolution and plasmid partitioning systems or a combination thereof [93,94]. Plasmid multimer structures are commonly occurring during replication and thus reduce the effective number of plasmid copies available for distribution to the daughter cell upon cell division. The presence of plasmid multimer resolution systems promotes a monomeric state of the plasmid within a cell and therefore facilitates plasmid segregation and stability. This process can additionally be enhanced if plasmid maintenance systems assist to actively distribute plasmid copies to all daughter cells during cell division [94–96].

Toxin/antidote systems encode genetic information for the production of a stable product mediating a toxic function and an unstable product acting as an antidote compensating the toxic function. The toxin element inhibits growth essential functions in the cell, but is neutralized in the presence of the antidote. Upon cell division, toxin and antidote components are propagated by the cytoplasm to all daughter cells where the toxin remains active, in contrast to the relatively unstable antidote. In case of plasmid loss the antidote cannot be produced anymore thus causing growth inhibition or death of plasmid-free cells, whereas cells containing a plasmid copy remain healthy [33,91,93]. Such toxin/antidote systems are already successfully applied to stabilize expression vectors including vectors for *R. eutropha* H16 [58,60]. The plasmid retention rates achieved with the application of such systems reached 95% over a period of 96 h without selection pressure [43].

One such system is the RP4 derived partitioning system, which encodes a toxin/antidote system, a plasmid multimer resolution system and further not yet fully characterized elements [31–33]. This system was originally shown to act as an extremely efficient stabilization system in *E. coli* and was further adapted to stabilize vectors based on pBBR1, RP4, RSF1010 and pSa replication elements in *R. eutropha* H16. Whereas vectors with RP4, RSF1010 and pSa replication elements exhibited no plasmid loss, vectors based on pBBR1 replication elements exhibited only 4% plasmid loss after cultivation for 96 h (repeated transfer every 24 h) without selection pressure [58,60] (Table 1).

The complementation of essential metabolic functions of auxotrophic strains represents another efficient approach to enhance plasmid maintenance. Examples related to *R. eutropha* H16 are relying on complementing mutants that lack essential genes required for growth under heterologous or lithoautotrophic conditions including genes like the hydrogenase transcription factor (*hoxA*), 2-keto-3-desoxy-6-phosphogluconate-aldolase (KDPG-aldolase; *eda*), xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (*xfp*) and pyrroline-5-carboxylate reductase (*proC*). Plasmid retention rates of 90% were obtained with such complementation systems during fermentation of plasmid-containing *R. eutropha* H16 strains [12,54,59,97] (Table 1).

As demonstrated with the model organism *R. eutropha* H16, the application of metabolism-based complementation systems or the use of toxin/antidote systems, multimer resolution systems and partitioning systems or a combination of these systems prove to be valuable concepts for constructing stable plasmid-based vectors for Gram-negative bacteria.

Plasmid transfer and mobilization

Transformation mediated by various physical or chemical treatments is the standard approach to transfer DNA into living cells when establishing recombinant organisms. However, especially with many Gram-negative bacteria such processes suffer from low efficiencies or are even not feasible, most probably caused by the complex cell envelope structure of this group of bacteria. Transfer of plasmid DNA from a donor to an acceptor cell is naturally accomplished by conjugation, a very efficient and common process in nature and thus representing an alternative to transformation [98]. Conjugative plasmid transfer was verified among Gram-negative bacteria, from Gram-negative to Gram-positive bacteria and even from bacteria to higher eukaryotes [28,99,100]. The essential elements required for conjugation are the transfer-genes (*tra*-genes) and a mobilization site (*mob*) including the origin of transfer (*oriT*). Hereby, two regions encoding the mating pair formation (Mfp) system and the DNA transfer and replication system (Dtr) are essential components [101]. A direct and stable cell-to-cell contact is established between the donor and recipient strain by forming a transmembrane pore that is stabilized by pili [102]. Subsequently, a single-stranded DNA copy, generated by rolling circle DNA replication, is transferred through the transmembrane pore and finally reconstituted and established in the new host [103,104]. The *tra*-genes can generally be provided *in trans* whereas the mob functions including *oriT* need to be present *in cis* on the transferred plasmid [105].

In laboratory practise, conjugative transfer of vectors is usually performed from *E. coli* to the desired host bacterium. It can be mediated either by a helper plasmid that is only able to replicate in *E. coli* containing the essential tra functions of RP4 or by using a specific *E. coli* strain with chromosomally integrated RP4 *tra*-genes (e.g. *E. coli* S17-1) [106,107]. As with many other bacterial species, transformation protocols (e.g. electroporation) yield unsatisfactory results with *R. eutropha* H16 and only conjugative DNA transfer is feasible. Recently determined mobilization efficiencies of plasmids containing mobilization sequences derived from broad-host-range plasmids pBBR1, RP4 and RSF1010 indicate that the interaction of *tra*-genes, *oriT* as well as the *mob* regions, is crucial for the process of conjugation. The results showed that mobilization efficiencies of RP4 and RSF1010 derived *mob* regions were 50,000 to 5000 times higher compared to the mobilization efficiencies obtained for the pBBR1 mob region. The differences in these values may be related to the efficiency of interaction among the RP4 transfer proteins and the particular mobilization apparatus [58] (Table 1).

Expression elements & Secretion signals

The design of expression systems in prokaryotic cells relies mainly on elements determining transcription efficiency (e.g. promoters, terminators, regulatory systems) and translation initiation (shine dalgarno sequences). A skilful combination of these elements can be used in order to create the desired expression system with respect to application and host compatibility [1,2]. In case of *R. eutropha* H16, a variety of functional promoters, shine dalgarno sequences and terminators were identified and successfully applied for expression studies [7,58,108]. The use of regulatory elements that enable the application of inducible systems is favourable as it provides the possibility to precisely regulate expression and thereby increase the product yield [58,108,7]. Secretion of the expressed polypeptide into the environment is another desired feature for recombinant protein production and represents a significant benefit for product recovery and purification [109–111]. Therefore mainly signal sequences from sec or tat secretion pathways are used to secret heterologous proteins in many prokaryotic organisms [111,112]. Linking a signal sequence and a protein intended for secretion exhibits a unique interaction which represents a key requirement for successful secretion of the protein of interest [113]. However, with *R. eutropha* H16 secretory expression of heterologous proteins has not yet been studied.

Conclusion

This review discusses important general aspects for the design of broad-host-range expression vectors in order to highlight essential features that assure a reliable application in various Gram-negative bacteria. The importance of single building blocks and their interaction with each other and host specific factors was illustrated using the β-proteobacteria *R. eutropha* H16 as an example. It could be demonstrated that a skilful combination of components from the genetic toolbox is the key for successful expression vector design and their application in Gram-negative bacteria outside the *E. coli* system, in order to make use of various interesting features of such organisms for efficient protein and metabolite production.

Authors' contributions

SG and PK wrote the initial manuscript and HS contributed some additional thoughts and formulations. All authors read, corrected and approved the final version.

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Chapter 3

3.3 Design of inducible expression systems for improved protein production in *Ralstonia eutropha* H16

Contribution to this Chapter

Planning experiments ~ 85%

Laboratory work ~ 95%

Writing manuscript ~ 85%

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Design of inducible expression systems for improved protein production in *Ralstonia eutropha* H16

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Abstract

Ralstonia eutropha H16 (*Cupriavidus necator* H16) is a Gram-negative, facultative chemolithoautotrophic bacterium which can use H_2 and CO_2 as sole energy and carbon sources in the absence of organic substrates. The biotechnological use of *R. eutropha* H16 on an industrial scale has already been established. However, one major problem encountered so far was the lack of inducible expression systems promoting suitable induction features under large scale conditions that do not rely on the adaption of growth conditions for induction. Within this study two inducible expression systems were designed on the basis of the strong *j5* promoter, derived from the bacteriophage T5, in combination with the *E. coli lacI* and the *Pseudomonas putida* cumate regulatory elements. Both systems display desired regulatory features and further increase the number of suitable inducible expression systems for the production of metabolites and proteins in *R. eutropha* H16.

Keywords: *Ralstonia eutropha* H16; Inducible expression system; Cumate; IPTG; Cre-LoxP

1. Introduction

Ralstonia eutropha H16 (now named *Cupriavidus necator* H16) is a facultative chemolithoautotrophic, soil-dwelling Gram-negative bacterium. The multi-replicon genome of *R. eutropha* H16 has a total size of 7,416,678 bp and consists of two chromosomes as well as a megaplasmid (pHG1) (Pohlmann et al., 2006). A versatile set of genes for energy and carbon metabolism is encoded within the genome of *R. eutropha* H16, which enables growth under heterotrophic, lithoautotrophic or organoautotrophic conditions (Cramm, 2009). Accordingly, a diversity of growth substrates are accepted by this bacterium including a number of organic acids such as acetic acid and sugars like fructose under heterotrophic growth conditions; which are metabolized via the Entner–Doudoroff (ED) pathway and the tricarboxylic acid (TCA) cycle (Cramm, 2009; Pohlmann et al., 2006). Moreover, CO_2 and H_2 can serve as the sole carbon and energy sources under lithoautotrophic growth conditions, respectively. In this case CO_2 is fixed via the Calvin-Benson-Bassham (CBB) cycle and H_2 oxidized by [NiFe]-hydrogenases (Bowien and Kusian, 2002). In a similar way *R. eutropha* H16 is also capable of growing organoautotrophically by splitting formic acid into CO_2 and NADH with the help of a membrane-bound formate dehydrogenase, which allows the bacterium to directly utilize NADH and fix the released CO_2 via the CBB cycle (Cramm, 2009; Grunwald et al., 2015). Based on this great diversity of accepted energy and carbon sources as well as the ability of *R. eutropha* H16 to synthesize large amounts of the biodegradable polymer polyhydroxybutyrate (PHB), widespread biotechnological interest in this bacterium has been developed (Atlić et al., 2011; Ewering et al., 2006).

Especially the application of *R. eutropha* H16 as a production host for PHB is well-established with fermentation processes on a large industrial scale (Kessler et al., 2001). However, the utilization of *R. eutropha* H16 as a production host is not limited to the synthesis of PHB or derivatives thereof. The ability of the organism to grow to high-cell-densities under lithoautotrophic or heterotrophic conditions further promotes the biotechnological application of *R. eutropha* H16 for the production of metabolites and proteins (Barnard et al., 2004; Lütte et al., 2012). Unlike *Escherichia coli*, *R. eutropha* H16 can be cultivated in high-cell-density fermentations without accumulating growth inhibiting organic acids. This offers great advantages for the fermentation process including higher product concentrations, increased productivity and improved operating costs (Andersson et al., 1994; Chen et al., 1992). Large scale high-cell-density fermentation processes employing *R. eutropha* H16 yielded cell densities up to 230 g/l and high amounts of the target protein (Barnard et al., 2004; Ryu et al., 1997). In addition to these features, the protein folding capacities of *R. eutropha* H16 enable the production of properly folded proteins under stress conditions with no significant inclusion body formation (Gruber et al., 2014; Srinivasan et al., 2002). In order to fully take advantage of the natural capabilities of *R. eutropha* H16 in biotechnological processes, natural

features need to be refined or newly established in the organism. One simple and efficient way to introduce such functions is provided by the use of expression plasmids.

The design of stable expression plasmids anticipated for the use in *R. eutropha* H16 requires adapted features related to gene expression, plasmid replication, plasmid stability and segregation (Gruber et al., 2015). The use of replication elements derived from broad-host-range plasmids such as RSF1010, RP4, pBBR1, pSa and the megaplasmid pMOL28 from *Ralstonia metallidurans* CH43 for instance promote successful replication of expression plasmids at different copy numbers in *R. eutropha* H16 (Ditta et al., 1985; Gruber et al., 2014; Kovach et al., 1995; Sato et al., 2013). However, in order to prevent considerable plasmid loss during the cultivation of *R. eutropha* H16, which occurs at a significant rate despite the use of antibiotic selection, systems promoting plasmid stability and maintenance need to be implemented. The use of plasmid multimer resolution, plasmid partitioning and toxin/antitdote addiction systems or metabolism-based addiction systems resulted in significant improvement of plasmid retention rates (Budde et al., 2011; Gruber et al., 2014; Sato et al., 2013; Voss and Steinbüchel, 2006).

A number of functionally active promoters and inducible expression systems were identified to efficiently control the expression of the particular gene of interest in *R. eutropha* H16. This includes heterologous promoters such as P_{lac}, P_{lacUV5}, P_{tac}, P_{BAD}, P_{T5} and P_{T7} as well as numerous native promoters derived from operons involved in pyruvate metabolism, PHB biosynthesis, acetoin metabolism and the *cbb* operon (Barnard et al., 2005; Bi et al., 2013; Delamarre and Batt, 2006; Fukui et al., 2011). In addition, a set of promoters derived from the genome of the bacteriophage T5 was shown to be highly active in *R. eutropha* H16 (Gruber et al., 2014). Several of these promoters were also used in combination with regulatory elements as inducible expression systems. Among these are heterologous expression systems based on the particular operator sites and repressor proteins including the TetR repressor responding to the inducer anhydrotetracycline (ATc) (Li and Liao, 2015), the AraC repressor and the inducer L-arabinose, the LacI repressor and inducer IPTG dependent on an integrated lactose permease (LacY) function and the inducible expression system based on the XylS repressor and the inducer m-toluic acid (Bi et al., 2013). Furthermore, a number of homologous inducible expression systems were characterized for the use in *R. eutropha* H16 on the basis of the *cbbL* promoter, which is induced under lithoautotrophic growth conditions (Lütte et al., 2012) and the *phaP* promoter, which is induced by phosphate depletion (Srinivasan et al., 2002). However, only a small number of inducible expression systems did function in a satisfactory manner or are applicable for large scale fermentation processes with *R. eutropha* H16 under a broad range of growth conditions. The inducible expression systems based on the *cbbL* and *phaP* promoters for example require specific adaptations of the fermentation process in order to create inducing

conditions, which constraints their use to fermentation processes that account for phosphate depletion or are performed under lithoautotrophic conditions (Lütte et al., 2012; Srinivasan et al., 2002). The use of TetR-based expression systems proved to show valuable induction features. Nonetheless, the use of tetracycline inducers in large scale fermentation processes is not feasible due to the antibiotic nature of the inducers (Li and Liao, 2015). The widely used LacI-based expression system was shown to work in *R. eutropha* H16 on the basis of an incorporated lactose permease (LacY) function. However, even though IPTG could be transported across the cellular membranes full induction of the applied promoter could not be obtained with this system so far (Bi et al., 2013). In comparison, tightly regulated and highly tunable expression was achieved by inducible expression systems based on the regulatory elements of the p-cumate (4-isopropylbenzoic acid) degrading operon derived from *Pseudomonas putida* F1 in several microorganisms and human cell lines. The cumate based inducible expression systems were found to function efficiently relying on passive transport of the non-toxic and comparatively cheap inducer p-cumate (Choi et al., 2010; Kaczmarczyk et al., 2013; Mullick et al., 2006). Accordingly, a cumate-induced expression system was designed for the use in *R. eutropha* H16 in this study.

The aim of this study was to extend and improve the range of inducible expression systems for the biotechnological application in *R. eutropha* H16. A total of two inducible expression systems were designed on the basis of the *j5* promoter in combination with the lac and cumate regulatory elements. Both systems exhibit desired regulatory features and increase the number of inducible expression systems for the production of metabolites and proteins in *R. eutropha* H16.

2. Materials and Methods

2.1 Strains, plasmids and primers

All strains and plasmids used in this study are listed in tables 1 and 2. Primers used for PCR amplifications are summarized in table S1 (supplementary data).

2.2 Cultivation of *E. coli* and *R. eutropha* H16 strains

E. coli S17-1 cells were cultivated at 37°C on lysogeny broth (LB) media with kanamycin [40 µg/ml] or chloramphenicol [25 µg/ml]. *R. eutropha* H16 cells were cultivated at 28°C using nutrient broth (NB) or tryptic soy broth (TSB) media supplemented with gentamicin [20 µg/ml], chloramphenicol [100 µg/ml] or kanamycin [200 µg/ml] and 0.6% or 2% fructose according to application. All basic media components were purchased from Sigma-Aldrich (St. Louis, MO, USA), Carl Roth (Arlesheim, Germany) and Becton Dickinson and Company (Franklin Lakes, NJ, USA).

2.3 DNA preparation

Standard procedures were used for PCR, DNA preparation and manipulation as well as genomic DNA isolation (Sambrook and Russel, 2001). Restriction enzymes and GeneJET Plasmid Miniprep Kits by Thermo Scientific (Waltham, MA, USA), Q5® High-Fidelity DNA Polymerase by New England Biolabs (Ipswich, MA, USA), T4 DNA Ligation reaction mixtures and Wizard® SV Gel and PCR Clean-Up System by Promega (Madison, WI, USA) and Easy-DNA™ Kit by Invitrogen (Carlsbad, California, USA) were used according to the manufacturer's protocols.

2.4 Plasmid construction

The plasmids pKRL-P_{j5}-egfp and pKRC-P_{j5}-egfp were constructed on the basis of the pKRSF1010-P_{tac}-egfp backbone (Gruber et al., 2014). Primers Pj5-laco-fwd1, Pj5-cyO-fwd-1 and KanR-SpeI-rev were used to amplify *egfp*, *rrnB* and Km^r from pKRSF1010-P_{tac}-egfp. Two subsequent PCR runs were performed with forward primers Pj5-lacO-fwd-2, Pj5-lacO-NotI-fwd-3 or Pj5-cyO-fwd-2, Pj5-cyO-NotI-fwd-3 and the reverse primer KanR-SpeI-rev to add the particular *lacO* or cumate operator sequences along with P_{j5} to the previously amplified PCR product. The final PCR products and pKRSF1010-P_{tac}-egfp were restricted with *Not*I/*Spe*I and combined by ligation. Co-expression

cassettes containing *cymR* and *lacI* were constructed by overlap extension PCR of DNA fragments coding for the constitutive promoter of the chloramphenicol resistance marker P_{Cmr}, *cymR* or *lacI* and a T7 terminator sequence. P_{Cmr} was amplified with primers CymR-P-fwd-SpeI and CymR-P-oe from the pSa plasmid, *cymR* was amplified from the genomic DNA of *Pseudomonas putida* F1 with primers CymR-gen-fwd-oe, CymR-gen-T7tt-rev-1 and CymR-gen-T7tt-rev-2-SpeI, *lacI* was amplified with primers LacI-SpeI-fwd and LacI-SpeI-rev from pMS470Δ8 and the T7 promoter sequence was encoded on the primers. Depending on application the co-expression cassettes were cloned into the particular plasmids via *Spe*I restriction sites to obtain pKRL-P_{j5}-egfp and pKRC-P_{j5}-egfp. The plasmid pKRC-P_{j5}-estA was created by combining the backbone of pKRC-P_{j5}-egfp restricted with *Xba*I/*Cla*I and *estA* cut *Xba*I/*Cla*I. *EstA* was derived from pKRSF1010-P_{tac}-Ru1 (Gruber et al., 2014). A description of the pINT_lacY_Phac_loxP plasmid design used for the construction of *R. eutropha* RS1 is attached in the supplementary data.

2.5 Plasmid transfer

Plasmids were transformed into *E. coli* cells using standard electroporation protocols (Sambrook and Russel, 2001). *E. coli* S17-1 transformants were used as a donor strain to transfer plasmids to *R. eutropha* H16 by conjugation (Srivastava et al., 1982). Conjugation was performed according to the protocol of Simon et al. (1983). The cell suspensions were plated out on TSB gentamicin [20 µg/ml] and kanamycin [200 µg/ml] or gentamicin [20 µg/ml] and chloramphenicol [100 µg/ml] agar plates for selection of *R. eutropha* H16 transconjugants.

2.6 Strain engineering

R. eutropha RS1 was constructed by the integration of the expression cassette P_{H16_B1772}*lacYCm*^{r} at the *phaC* (*H16_A1437*) locus followed by recycling the Cm^r marker. Therefore the plasmid pInt_lacY_phaC_loxP was transferred to *R. eutropha* H16 by conjugation. After integration at the *phaC* locus the excision of the resistance marker by the Cre-loxP system was induced with p-cumate in the integration strain *R. eutropha* H16 Δ*phaC*ΩP_{H16_B1772}*lacYCm*^{r}. This was accomplished by selection on TSB gentamicin [20 µg/ml] and p-cumate [20 µg/ml] agar plates following plasmid transfer by conjugation from *E. coli* S-17 carrying the plasmid pCM_Cre coding for the Cre recombinase.

2.7 Fluorescence unit measurement

ONCs of *R. eutropha* H16 transconjugants were grown in liquid TSB kanamycin [200 µg/ml] media and used to inoculate TSB media to an OD_{600} of 0.2. The cultures were grown to an OD_{600} of approximately 0.8 when they were induced with 30 µM, 60 µM or 120 µM p-cumate and 0.01 mM, 0.1 mM or 1 mM IPTG. Afterwards samples were taken every 2 hours and eGFP expression levels were determined based on fluorescence unit measurements with FLUOstar Omega (BMG Labtech, Ortenberg, Germany) at excitation wavelength of 480 nm and emission wavelength of 510 nm. Fluorescence units (FU) were determined for *R. eutropha* H16 transconjugants and related to the OD_{600} values of the culture to obtain relative fluorescence units (RFU). In either case the RFU values of all samples were related to *R. eutropha* H16 (pKRSF1010Δegfp), which served as negative control.

2.8 Quantification of esterase activity

The photometric esterase activity assay based on the substrate p-nitrophenyl butyrate (Sigma-Aldrich) was performed as previously described by Gruber et al. (2014).

2.9 SDS-PAGE and Western Blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 4–12% NuPAGE® Bis-Tris Gel (Invitrogen) to separate proteins of the whole cell extract. In each case 10 µg of total protein or 0.2 OD_{600} units were added per lane. Transfer of proteins to a Roti-NC HP40.1, 0.2 µm nitrocellulose membrane (Carl Roth) was accomplished with the TE22 Mini Transfer Tank Unit (Hoefer Inc., Holliston, MA, USA) according to manufacturer's recommendations. A primary mouse antibody (Monoclonal anti GFP, G6795; Sigma-Aldrich) and a horseradish peroxidase linked secondary goat-anti-mouse antibody (Sigma-Aldrich) were used for eGFP detection on a nitrocellulose membrane. A primary rabbit antibody (Monoclonal anti HIS; D3I10; NEB) and a horseradish peroxidase linked secondary anti-rabbit antibody (NEB) were used for HIS detection on a nitrocellulose membrane. Proteins were visualized using SuperSignal (Pierce, Rockford, USA).

3. Results and Discussion

3.1 Setup of *lacI* and *cymR* based inducible expression systems

Several promoter sequences derived from the bacteriophage T5 were previously shown to be highly active in *R. eutropha* H16 (Gruber et al., 2014). Out of these, the *j5* promoter was selected for the characterization of the IPTG and cumate-induced expression systems in *R. eutropha* H16. A set-up was chosen on the basis of a pKRSF1010 backbone in which the j5 promoter was used to drive the expression of the reporter genes *egfp* or *estA*. The pKRSF1010 vector backbone encodes next to the RSF1010 *oriV* and *mob* sequences, a RP4 partitioning system, which includes a site specific recombination system and a toxin/antitoxin system in order to significantly increase plasmid stability and propagation in *R. eutropha* H16 (Gruber et al., 2014). All regulatory and functional elements of the IPTG or cumate-induced expression cassettes were organized identically: P_{j5}, followed by the particular operator sites, a Shine-Dalgarno sequence and the genes to be expressed (*egfp* or *estA* in this study) (Figure 1). The expression cassettes containing the repressor genes *lacI* and *cymR* were included in the particular pKRSF1010 backbone. Both repressor genes were expressed from a weak constitutive promoter, P_{cmr}, derived from the chloramphenicol resistance gene of the pSa plasmid.

3.2 Construction of the *lacY* containing *R. eutropha* strain RS1

In order to enable the use of the IPTG-induced expression system in *R. eutropha* H16 an IPTG transport function had to be integrated additionally, since this kind of transport cannot be accomplished naturally. Therefore, the *E. coli* derived lactose permease gene (*lacY*; JF300162.1) was engineered to be under the control of the constitutive *H16_B1772* promoter derived from *R. eutropha* H16 (Gruber et al., 2014). The expression cassette containing *lacY* was integrated into the *phaC* (*H16_A1437*) locus on chromosome 1 of *R. eutropha* H16 and the chloramphenicol resistance marker was subsequently removed by the Cre-loxP system to obtain *R. eutropha* RS1. Unlike previously reported for a similar IPTG-induced expression system that was designed for an application in *R. eutropha* H16 (Bi et al., 2013), the integrated lactose permease function in the current study did enable sufficient IPTG transport across the cells' membranes and triggered the induction of the *j5* promoter completely (Table S2).

3.3 Characterization of the *lacI* based inducible expression system

Characterization of the IPTG-induced expression system was performed on the basis of *R. eutropha* RS1 (pKRL-P_{j5}-egfp) and RFU were determined to monitor the expression of *egfp* over a time period of 9 hours and at 24 hours after induction. Furthermore, protein expression data were obtained on basis of SDS-PAGE and Western Blot analysis. *R. eutropha* RS1 (pKRL-P_{j5}-egfp) cultures were induced 3 hours after inoculation at an OD_{600} of approximately 0.8 with 0.01 mM, 0.1 mM or 1 mM of IPTG. *R. eutropha* RS1 (pKRL-P_{j5}-egfp) cultures induced with a concentration of 0.01 mM IPTG exhibited increasing fluorescence values from 2500 RFU to 5500 RFU at 2 hours and 6 hours after induction, respectively. After 24 hours the measured fluorescence had increased to 20400 RFU. A tenfold increased inducer concentration of 0.1 mM IPTG resulted in 4500 RFU after 2 hours, 11000 RFU after 6 hours and 33100 RFU after 24 hours. An IPTG induction concentration of 1 mM triggered strong eGFP expression, corresponding to 5200 RFU, 13000 RFU and 36100 RFU after 2, 6 and 24 hours after induction, respectively. Moreover, the RFU values obtained for the uninduced cultures of *R. eutropha* RS1 (pKRL-P_{j5}-egfp) did also increase steadily over time, from 600 RFU after 3 hours to 1500 RFU after 9 hours and 5400 RFU at 24 hours after inoculation. The obtained RFU values and eGFP expression data (Figure 2A and Figure S1) for the uninduced *R. eutropha* RS1 (pKRL-P_{j5}-egfp) cultures, revealing significant eGFP expression, do most likely result from remaining activity of the comparatively strong *j5* promoter. This does presumably result from the weak interactions of the LacI repressor protein and the lac operator DNA sequence (Penumetcha et al., 2010), which allows for leaky eGFP expression in significant amounts in uninduced *R. eutropha* RS1 (pKRL-P_{j5}-egfp) cultures, despite the use of two consecutive lac operator sequences. In induced cultures of *R. eutropha* RS1 (pKRL-P_{j5}-egfp) the rapid and strong induction of eGFP expression in response to IPTG did significantly decrease the growth of the culture (Figure 2B). Generally, higher concentrations of the inducer IPTG resulted in enhanced eGFP formation and strongly decreased culture growth; however, the level of eGFP production did not directly correlate with the amount of IPTG applied. A stepwise increase in inducer concentration by a factor of ten from 0.01 mM IPTG to 0.1 mM IPTG to 1 mM IPTG did yield RFU values of 5500 RFU, 11000 RFU and 12600 RFU after 6 hours of induction, respectively. The comparatively minor increase observed in RFU values for the cultures induced with 0.1 mM IPTG and 1 mM IPTG could result either from a limitation in the IPTG transport capacity of the lactose permease or fully induced *j5* promoter activity that is already reached at an inducer concentration of approximately 0.1 mM IPTG. Consequently, a stepwise increase in IPTG inducer concentration by a factor of 10 does not trigger a steady increase in eGFP expression levels, accordingly.

Nevertheless, a large amount of eGFP was produced in *R. eutropha* RS1 (pKRL-P_{j5}-egfp) in a short amount of time despite the significant decline of growth in the induced cultures. Moreover, a high level of eGFP expression was maintained for at least 24 hours in all induced cultures at a low growth rate (see Figure 2 and Table S2). Results of previous studies examining the induction pattern of IPTG-induced expression systems with flow-cytometry experiments on the basis of *E. coli* cultures, revealed great differences in the strength of induced expression levels of individual cells across the entire culture (Choi et al., 2010). An unequal distribution of the inducer IPTG due to the active transport across the cells' membranes resulted in very heterologously occurring IPTG-based expression across the population. Moreover, in a significant number of cells expression was highly induced and caused cell lysis (Choi et al., 2010).

3.4 Characterization of the *cymR* based inducible expression system

In comparison to the IPTG-induced expression system, the cumate-induced expression system does not require active transport of the inducer p-cumate. The inducer diffuses through the membrane and triggers a smooth and steady expression of the gene of interest across the entire culture (Choi et al., 2010). The characterization of the cumate-induced expression system was performed on the basis of *R. eutropha* H16 (pKRC-P_{j5}-egfp). In accordance with the measurements performed for the IPTG-induced expression system, cumate-induced *egfp* expression was observed over a time period of 9 hours and once 24 hours after induction (see Figure 3A and Table S2). EGFP expression was induced in *R. eutropha* H16 (pKRC-P_{j5}-egfp) cultures with 30 µM, 60 µM or 120 µM p-cumate 3 hours after inoculation at an OD_{600} of approximately 0.8. Testing different inducing concentrations, a concentration of 120 µM p-cumate was found to be sufficient to induce maximum expression. The induction of eGFP expression in *R. eutropha* H16 (pKRC-P_{j5}-egfp) cultures with a concentration of 30 µM p-cumate did increase fluorescence values from 500 RFU at 2 hours to 1600 RFU at 6 hours and 19400 RFU at 24 hours after induction. Induction with a concentration of 60 µM p-cumate resulted in fluorescence units of 800 RFU after 2 hours, 2200 RFU after 6 hours and 20700 RFU after 24 hours. An induction concentration of 120 µM triggered steady eGFP expression from 1100 RFU, 2700 RFU and 21200 RFU after 2, 6 and 24 hours, respectively. The RFU values obtained for the uninduced *R. eutropha* H16 (pKRC-P_{j5}-egfp) cultures on the other hand did increase slightly from 230 RFU after 2 hours to 640 RFU after 24 hours. The induction of *egfp* expression with different concentrations of p-cumate did strongly depend on the amount of inducer applied and enabled highly tunable expression characteristics. Moreover, the induction of expression did not occur as quick and intense as seen for the IPTG-induced expression system, but increased slowly and steadily over time. This is most likely a result of the diffusion process of the inducer through the

membranes of *R. eutropha* H16 and appears to be significantly slower in comparison to other bacteria such as *E. coli* (Choi et al., 2010). However, the slow uptake of p-cumate by *R. eutropha* H16 enabled continuous cell growth at a higher rate that yielded OD_{600} values of approximately 19 after 24 hours for all cumate-induced cultures (Figure 3B). In comparison, the IPTG-induced cultures grew slowly to approximately a third of the cell density; however, yielding eGFP expression in a comparable range after 24 hours (Table S2). Furthermore, the cumate-induced expression system was strongly repressed and remaining promoter activity was determined to be 650 RFU after 24 hours in comparison to 5500 RFU that were obtained for the IPTG-induced expression system after the same time (Figure 2, 3 and Table S2). The tight regulation of eGFP expression is most likely based on the strong interaction of the cumate repressor and operator sequences, which does not allow for significant promoter activity in an uninduced state (Choi et al., 2010; Kaczmarczyk et al., 2013). Furthermore, the inducer p-cumate is significantly cheaper compared to IPTG, which represents a crucial economic advantage concerning large scale fermentations.

3.5. Production of esterase EstA in *R. eutropha* H16

Esterase EstA derived from *Rhodococcus ruber* was additionally used as a model protein to analyze the capacity of the IPTG- and cumate-induced expression systems in *R. eutropha* H16. Since expression plasmids containing constitutive expression cassettes based on the *j5* promoter and *estA* could not be assembled, most likely due to significant stress of constitutive expression, the *estA* gene was cloned into the IPTG- and cumate-based inducible expression systems to obtain plasmids pKRC-P_{j5}-estA and pKRL-P_{j5}-estA. However, after induction with different concentrations of IPTG, EstA activity or protein could not be detected anymore in *R. eutropha* RS1 (pKRL-P_{j5}-estA) cultures. Sequencing of pKRL-P_{j5}-estA plasmids obtained after induction from the cultures did reveal deletion or insertion events in the promoter region, Shine-Dalgarno sequence or *estA* (data not shown). These events did most likely cause an arrest of *estA* expression in response to significant stress due to the rapid and strong induction by the IPTG-induced expression system. Unlike extensively engineered *E. coli* strains, with respect to knocked-out recombinase A or deleted transposon functions, the wild-type strain *R. eutropha* H16 used in this experimental setup does most likely still contain a number of such functions. On the contrary, the induction of expression in *R. eutropha* H16 (pKRC-P_{j5}-estA) with 120 µM p-cumate resulted in the formation of significant amounts of active EstA, with an activity of 6 U/mg, over a time period of 24 hours indicating that an induction occurring slowly and steadily over time seems to be beneficial for the expression of more complex proteins (Figure 4). Accordingly, the tightly regulated cumate expression system represents a valuable alternative regarding the expression of complex or even toxic proteins in *R. eutropha* H16.

4. Conclusion

In this study the design of inducible expression systems that are suitable for the use in *R. eutropha* H16 under large scale conditions is described. The inducible expression systems constructed on the basis of *lacI* and *cymR* regulatory elements do not require an adaptation of fermentation processes to provide induction conditions and share promising features such as tight regulation or highly tunable expression. Furthermore, significant amounts of the protein of interest were produced after a relatively short time of induction, including the production of more complex proteins. Altogether, it could be demonstrated that both inducible expression systems share valuable features that further promote the use of *R. eutropha* H16 for biotechnological applications including the production of metabolites and proteins.

5. Authors' Contributions

SG, PH, HS planned and started the project. Wet laboratory work was carried out by SG, JH, ZM, DS, ET and PH. SG and PH wrote the manuscript. HS contributed some additional thoughts. All authors read, corrected and approved the final version.

6. Acknowledgment

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Figure(s)

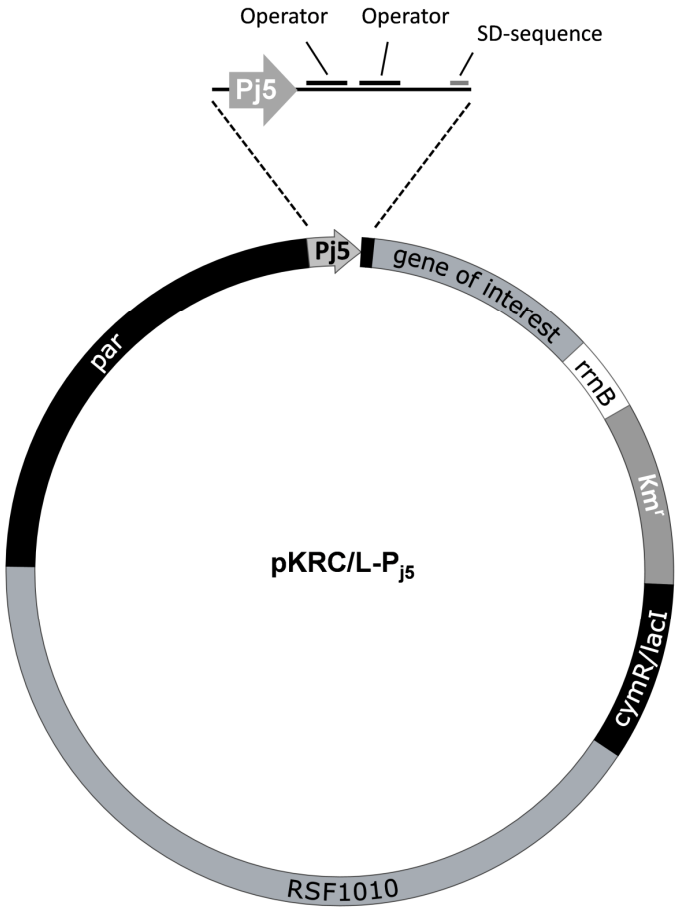

Figure 1: Illustration of the basic plasmid design. The plasmid backbone of pKRL-P_{j5} or pKRC-P_{j5} encode the terminator *rrnB*, the RP4 partition region *par,* a gene of interest (*estA* or *egfp*), the kanamycin resistance Km^r, the *j5* promoter, the RSF1010 *mob* and *oriV* sequences and the genes coding for the LacI or CymR repressor proteins as well as the particular operator sequences according to the inducible expression system in use.

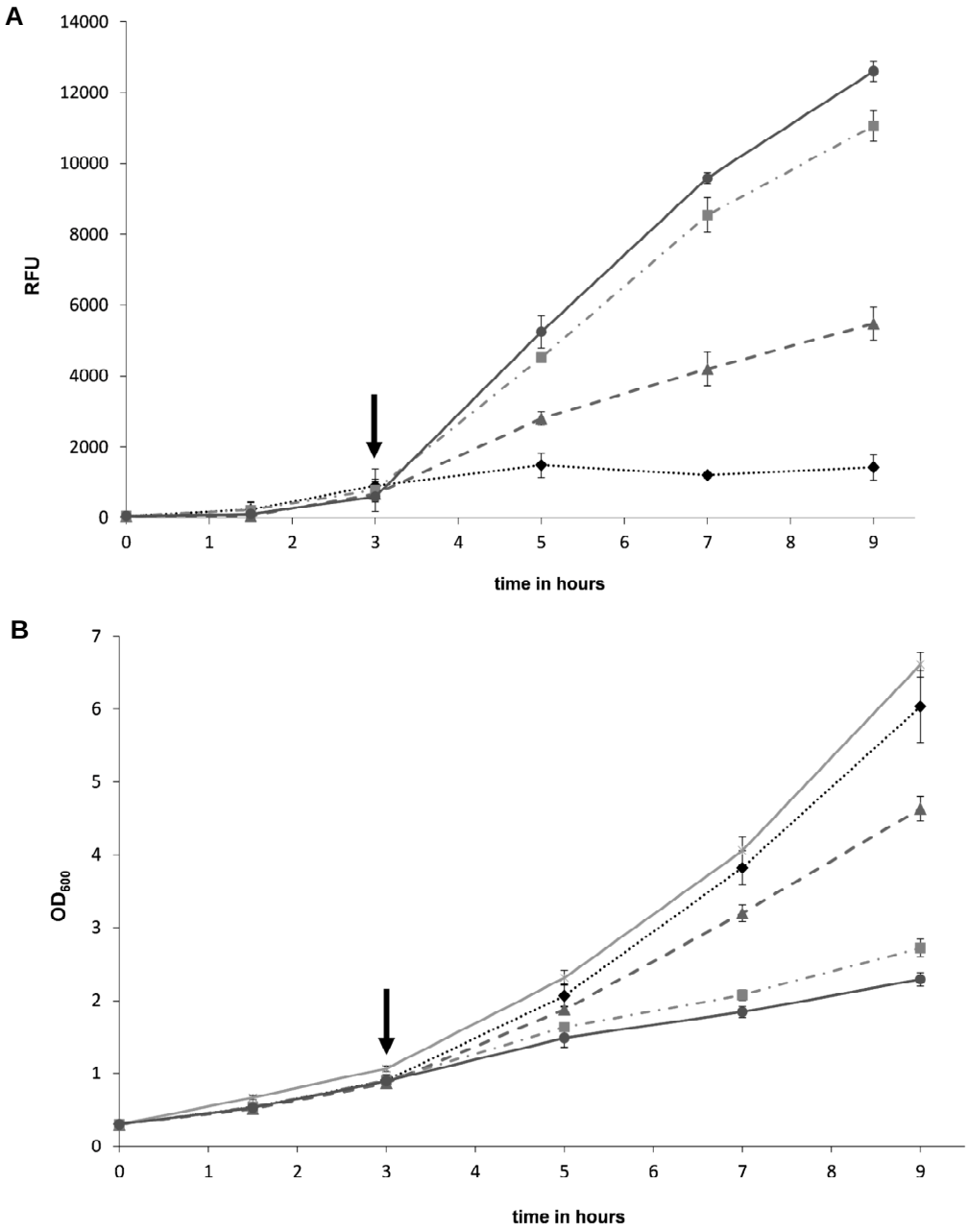

Figure 2: Time course of IPTG-induced eGFP expression (A) and growth (B) based on *R. eutropha* RS1 (pKRL-P_{j5}-eGFP). (A) *R. eutropha* RS1 transconjugants harboring plasmids pKRL-P_{j5}-eGFP, the point of induction is indicated by a black arrow. Uninduced cultures are labeled with black diamonds. Cultures induced with 0.01 mM IPTG (grey triangles), 0.1 mM IPTG (grey squares) and 1 mM IPTG (black circles). **(B)** The sample labels are identical to (A). Moreover, the solid grey line refers to the empty vector control *R. eutropha* H16 (pKRSF1010Δegfp)

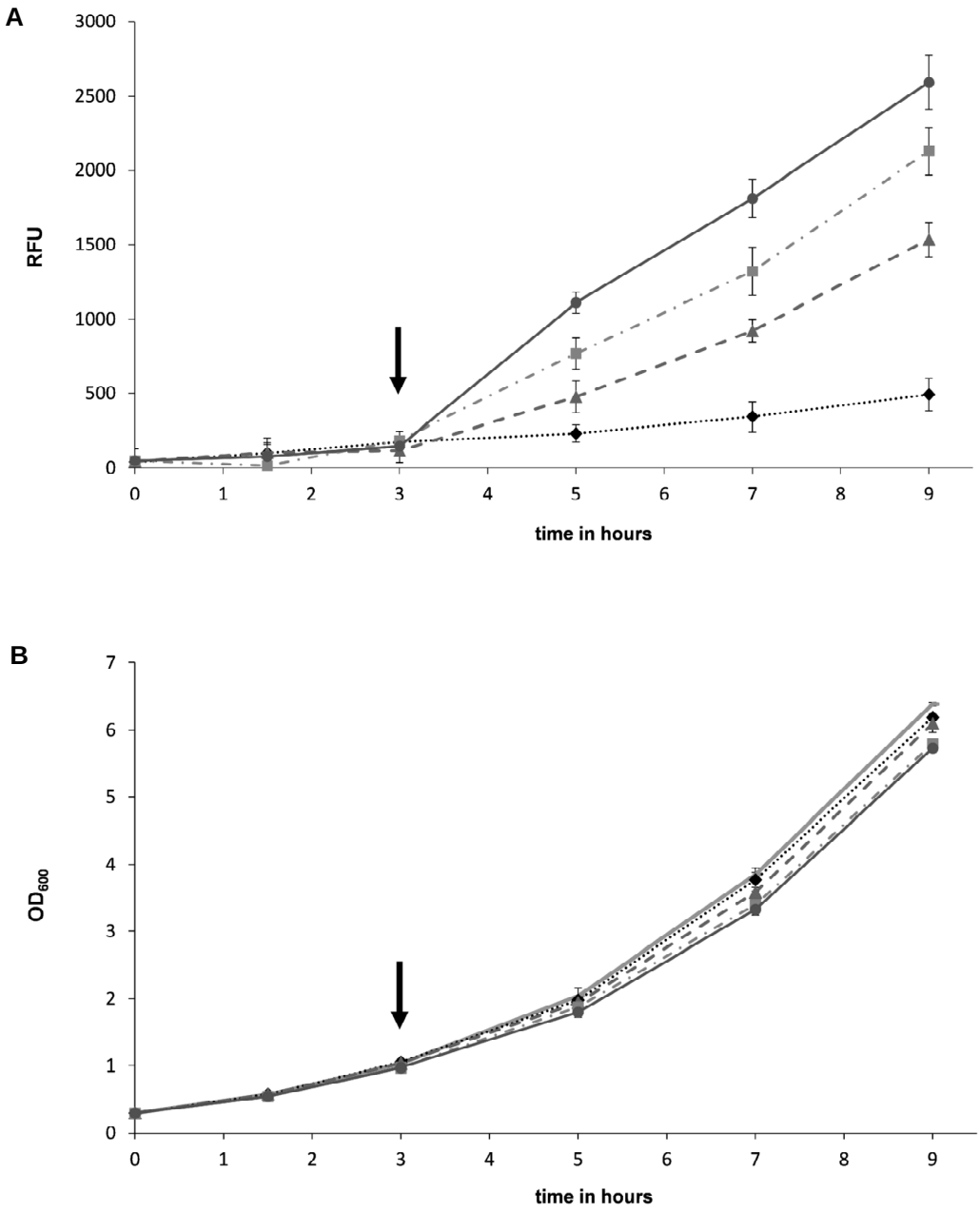

Figure 3: Time course of cumate-induced eGFP expression (A) and growth (B) based on *R. eutropha* H16 (pKRC-P_{j5}-eGFP). (A) *R. eutropha* H16 transconjugants harboring plasmids pKRC-P_{j5}-eGFP, the point of induction is indicated by a black arrow. Uninduced cultures are labeled with black diamonds. Cultures induced with 30 µM p-cumate (grey triangles), 60 µM p-cumate (grey squares) and 120 µM p-cumate (black circles). **(B)** The sample labels are identical to (A). Moreover, the solid grey line refers to the empty vector control *R. eutropha* H16 (pKRSF1010Δegfp)

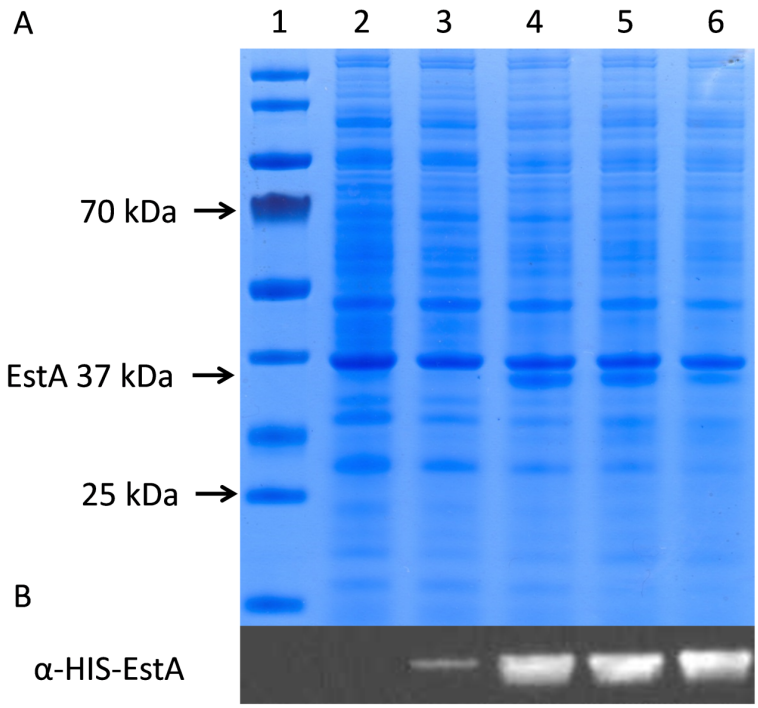

Figure 4: SDS-PAGE (A) and Western Blot (B) illustrating Cumate-induced EstA expression in *R. eutropha* H16 (A) SDS-PAGE of whole cell lysates of *R. eutropha* H16 (pKRC-P_{j5}-estA). Lane 1: PageRuler Prestained Protein Standard (Fermentas), Lane 2: *R. eutropha* H16 (pKRSF1010Δegfp), Lane 3: *R. eutropha* H16 pKRC-P_{j5}-estA at induction, Lane 4: *R. eutropha* H16 pKRC-P_{j5}-estA 8 hours after induction, Lane 5: *R. eutropha* H16 pKRC-P_{j5}-estA 24 hours after induction, Lane 6: *R. eutropha* H16 pKRC-P_{j5}-estA 32 hours after induction. **(B)** Western Blot of whole cell lysates of *R. eutropha* H16 (pKRC-P_{j5}-estA). Polyhistidine-tagged EstA was detected with a monoclonal anti-his antibody (α-HIS). The samples were applied in the same order as in (A).

Table(s)

Table1: Strains used in this study

Strain	Description	References or Source
E. coli MG1655	F^- λ^- *ilvG- rfb-50 rph-1*	Invitrogen
E. coli S17-1	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7 integrated into the chromosome	Invitrogen
E. coli TOP10	F´(*proAB, lacIq*, *lacZ*ΔM15, Tn10(tet-r)), *mcrA*, Δ(mrr-hsdRMS-mcrBC), Φ80ΔlacZΔM15, Δ*lacX74*, *deoR*, *recA1*, *araD139*(*ara, leu*), 7697, *galU*, *galK*, λ-, *rpsL*(streptomycin-r), *endA1*, *nupG*	Invitrogen
Pseudomonas putida F1	wildtype	DSMZ 6899[a]
R. eutropha H16	wildtype	DSMZ 428[a]
R. eutropha RS1	H16 ΔphaCΩPH16_B1772lacY	this study

Table 2: Plasmids used in this study

Plasmids		
RP4	broad-host-range plasmid, IncP	(Pansegrau et al., 1994)
pSa	broad-host-range plasmid, IncW	(Tait et al., 1982)
pMS470Δ8	Ap^r, P_{tac}	(Balzer et al., 1992)
pMS470Ru1	Ap^r, P_{tac}, *estA*	(Schwab et al., 2003)
pK470MobRP4	Km^r, P_{tac}, *mob*, *colE1*	this study

pInt_lacY_phaC	pK470MobRP4, *lacY* gene, $P_{H16\_B1772,}$ two phaC homologous regions	this study
pInt_lacY_phaC_loxP	pK470MobRP4, *lacY* gene, $P_{H16\_B1772,}$ two phaC homologous regions, loxP sites	this study
pCM_Cre	Cm^r, P_{tac}, *mob, colE1, cre, cymR*	this study
pKRSF1010-P_{j5}-egfp	Km^r, P_{j5}, *egfp*, *par*, RSF1010 *mob* and origin of replication	(Gruber et al., 2014)
pKRSF1010Δegfp	Km^r, P_{tac}, *par*, RSF1010 *mob* and origin of replication, deleted *egfp*	(Gruber et al., 2014)
pKRL-P_{j5}-egfp	Km^r, P_{j5}, *egfp*, *par*, *lacI*, RSF1010 *mob* and origin of replication	this study
pKRC-P_{j5}-egfp	Km^r, P_{j5}, *egfp*, *par*, *cymR*, RSF1010 *mob* and origin of replication	this study
pKRC-P_{j5}-estA	Km^r, P_{j5}, *estA*, *par*, *cymR*, RSF1010 *mob* and origin of replication	this study

[a] DSMZ, Deutsche Sammlung für Mikroorganismen und Zellkulturen.

[b] Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; *par*, RP4 site specific partitioning system

Supplemental Figures and Tables

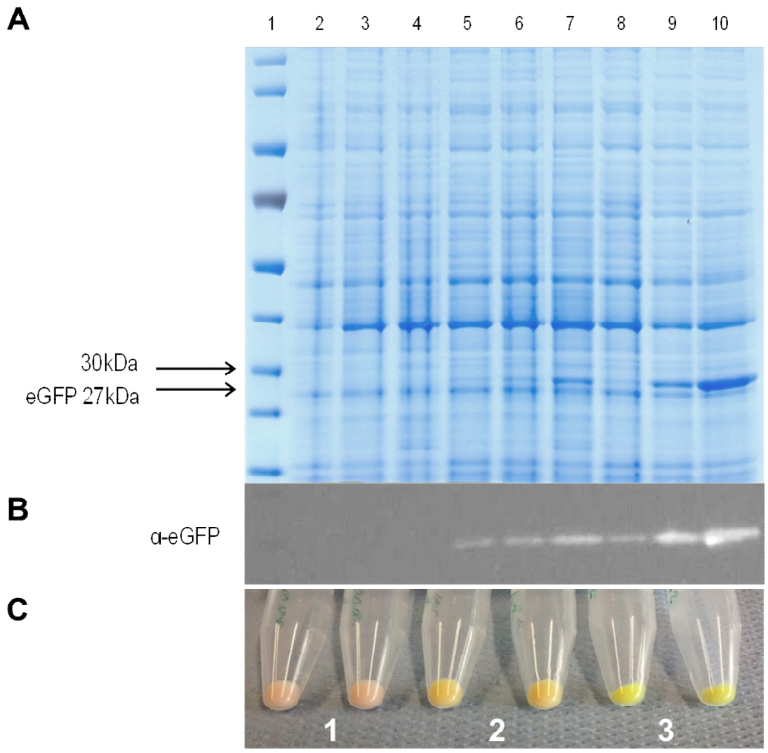

Figure S1: IPTG-induced eGFP expression in *R. eutropha* RS1 (pKRL-P_{j5}-egfp) illustrated on the basis of SDS-PAGE (A), Western Blot (B) and pelleted cells (C). EGFP expression was induced with 0.1 mM IPTG at an OD_{600} of 0.8 (A) SDS-PAGE of whole cell lysates of *R. eutropha* H16 (pKRSF1010Δegfp) and *R. eutropha* RS1 (pKRL-P_{j5}-egfp): Lane 1: PageRuler Prestained Protein Standard (Fermentas), Lane 2: *R. eutropha* H16 (pKRSF1010Δegfp) at the point of induction, Lane 3: *R. eutropha* H16 (pKRSF1010Δegfp) 8 hours after induction, Lane 4: *R. eutropha* H16 (pKRSF1010Δegfp) 24 hours after induction, Lane 5: Uninduced culture of *R. eutropha* RS1 (pKRL-P_{j5}-egfp) at the point of induction, Lane 6: Uninduced culture of *R. eutropha* RS1 (pKRL-P_{j5}-egfp) 8 hours after induction, Lane 7: Uninduced culture of *R. eutropha* RS1 (pKRL-P_{j5}-egfp) 24 hours after induction, Lane 8: Induced culture of *R. eutropha* RS1 (pKRL-P_{j5}-egfp) at the point of induction, Lane 9: Induced culture of *R. eutropha* RS1 (pKRL-P_{j5}-egfp) 8 hours after induction, Lane 10: Induced culture of *R. eutropha* RS1 (pKRL-P_{j5}-egfp) 24 hours after induction. **(B)** Western Blot of whole cell lysates of *R. eutropha* H16 (pKRSF1010Δegfp) and *R. eutropha* RS1 (pKRL-P_{j5}-egfp). EGFP was detected with a monoclonal anti-GFP antibody (α-eGFP). The samples were applied in the same order as in (A). **(C)** Cell pellets 24 hours after induction of the culture 1: *R. eutropha* H16 (pKRSF1010Δegfp), 2: *R. eutropha* RS1 (pKRL-P_{j5}-eGFP) undinduced and 3: *R. eutropha* RS1 (pKRL-P_{j5}-eGFP) induced with 0.1 mM IPTG.

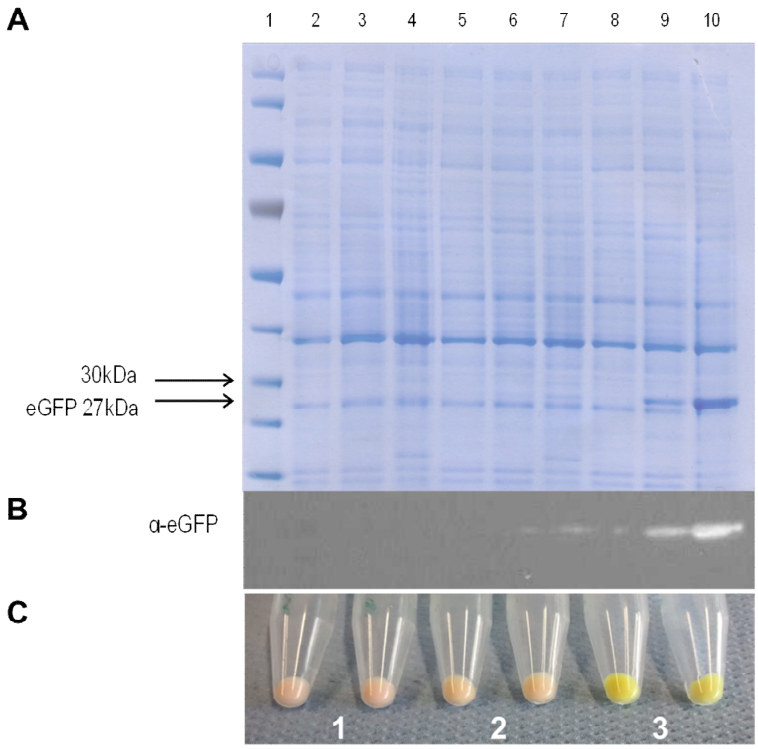

Figure S2: Cumate-induced eGFP expression in *R. eutropha* H16 (pKRC-P_{j5}-egfp) illustrated on the basis of SDS-PAGE (A), Western Blot (B) and pelleted cells (C). EGFP expression was induced with 120 µM p-cumate at an OD_{600} of 0.8 (A) SDS-PAGE of whole cell lysates of *R. eutropha* H16 (pKRSF1010Δegfp) and *R. eutropha* H16 (pKRC-P_{j5}-egfp): Lane 1: PageRuler Prestained Protein Standard (Fermentas), Lane 2: *R. eutropha* H16 (pKRSF1010Δegfp) at the point of induction, Lane 3: *R. eutropha* H16 (pKRSF1010Δegfp) 8 hours after the induction, Lane 4: *R. eutropha* H16 (pKRSF1010Δegfp) 24 hours after induction, Lane 5: Uninduced culture of *R. eutropha* H16 (pKRC-P_{j5}-egfp) at the point of induction, Lane 6: Uninduced culture of *R. eutropha* H16 (pKRC-P_{j5}-egfp) 8 hours after induction, Lane 7: Uninduced culture of *R. eutropha* H16 (pKRC-P_{j5}-egfp) 24 hours after induction, Lane 8: Induced culture of *R. eutropha* H16 H16 (pKRC-P_{j5}-egfp) at the point of induction, Lane 9: Induced culture of *R. eutropha* H16 (pKRC-P_{j5}-egfp) 8 hours after the point of induction, Lane 10: Induced culture of *R. eutropha* H16 (pKRC-P_{j5}-egfp) 24 hours after the point of induction. **(B)** Western Blot of whole cell lysates of *R. eutropha* H16 (pKRSF1010Δegfp) and *R. eutropha* H16 (pKRC-P_{j5}-egfp). EGFP was detected with a monoclonal anti-GFP antibody (α-eGFP). The samples were applied in the same order as in (A). **(C)** Cell pellets 24 hours after induction of the culture 1: *R. eutropha* H16 (pKRSF1010Δegfp), 2: *R. eutropha* H16 (pKRC-P_{j5}-eGFP) undinduced and 3: *R. eutropha* H16 (pKRC-P_{j5}-eGFP) induced with 120 µM *p*-cumate.

Table S1: List of primers used in this study

Primer	Primer Sequence 5' to 3'
H16_1776 rev	GTTTGTGTTTTTTAAATAGTACATGATTGGCTTCCTCGAGAG
H16_1776 SphI Fwd	ACATGCATGCTCAACAGCGACGAATACAGCAC
lacY fwd	GCTCTAGAATGTACTATTTAAAAAACACAAACTTTTGG
lacY rev	CCCAAGCTTTTAAGCGACTTCATTCACCTG
CmR_rev_XmaI	CCCGGGTAACTGGCCTCAGGCATTTGAG
phaC1_2_fwd_loxP_ XmaI	CCCGGGATAACTTCGTATAATGTATGCTATACGAAGTTATACATCGAGC ACGCGGCCATC
CmR_fwd_StuI_loxP	AGGCCTATAACTTCGTATAATGTATGCTATACGAAGTTATTTTGCGTTTC TACAAACTC
Cre_fwd_NdeI	CATATGTCCAATTTACTGACCGTAC
Cre_rev_HindIII	AAGCTTCTAATCGCCATCTTCCAGC
phaC1_1 Fwd SpeI	CTAGACTAGTATGGCGACCGGCAAAG
phaC1_1 Rev SpeI	CTAGACTAGTAGTCGTCCCAGGTGCTGC
phaC1_2 Fwd XhoI	CCGCTCGAGACATCGAGCACGCGGC
phaC1_2 Rev XhoI	CCGCTCGAGTCATGCCTTGGCTTTGACGTAT
pK470_fwd_SpeI	ACTAGTCAGGCAGCCATCGGAAGCTGTGG
pK470_rev_PstI	CTGCAGCGTTCGGCTGCGGCGAGCGGTA
MOB oriT RP4 Fwd	ACTAGTTCGATCTTCGCCAGCAGG
MOB oriT RP4 Rev	CTGCAGTCGACATCCGCCCTCAC
phaC1 Rev SpeI SphI	GGCATGCACTAGTAGTCGTCCCAGGTGCTG
HindIII_rrnB	AAGCTTGGCTGTTTTGGCGGATGAGAG
colE1 pK470 Fwd	TCGTTCCACTGAGCGTCAGA
rrnB pK470 Rev	TTCATGAGCGGATACATATTTGAATG
Fwd cmR NotI	GCGGCCGCTCATGACGAATAAATACCTGTGAC
Rev cmR SpeI	ACTAGTTAACTGGCCTCAGGCATTT
PTac-CymO-1	TGTGTGGAACAAACAGACAATCTGGTCTGTTTGTATTATAGAATTCGAG CTCGGTACC

PTac-CymO-2	AAGCGGCCGCAATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAACAAAC
Ptac-Cymo-overlap-	GTTATGCTAGGCGGCCGCAATGAGCTGTTG
Ptac-Cymo-overlap-Cre-rev	TGGACATATGTATATCTCCTTCTTAAAGTT
Cre-overlap-Ptac-CymO-fwd	AGGAGATATACATATGTCCAATTTACTGAC
Cre-overlap-rrnb+SmiI- rev	ACAGCCATTTAAATAAGCTTCTAATCGCCATCTTC
Rrnb-overlap-Cre+SmiI fwd	AAGCTTATTTAAATGGCTGTTTTGGCGGATGAGAG
MobRP4-overlap CymR+SmiI rev	TATTTAAATTCGACATCCGCCCTCACCGCCAG
CymR-overlap-MobRP4+SmiI fwd	GAATTTAAATACGGATGGCCTTTTTGCGTTTCTA
CymR-overlap-Ptac-rev	TTGCGGCCGCCTAGCATAACCCCTTGGGGCCTC
Pj5-lacO-fwd-1	GGATAACAATTCGATTCGGAATTGTGAGCGGATAACAATTCAATTCGAGCTCGGTACCCG
Pj5-lacO-fwd-2	ATTGACACAGGTGGAAATTTAGAATATACTGGGAATTGTGAGCGGATAACAATTCGATTC
Pj5-lacO-NotI-fwd-3	GCGGCCGCAAAAACCGTTATTGACACAGGTGGAAA
Pj5-cyO-fwd-1	ATAGATTCAACAAACAGACAATCTGGTCTGTTTGTATTATAAATTCGAGCTCCGTACCCG
Pj5-cyO-fwd-2	TTTAGAATATACTGAACAAACAGACAATCTGGTCTGTTTATCTTATAGATTCAACAAACA
Pj5-cyO-NotI-fwd-3	GCGGCCGCAAAAACCGTTATTGACACAGGTGGAAATTTAGAATATACTGAACA
KanR-SpeI-rev	CGGACTAGTGTCTGACGCTCAGTGGAACGAA
CymR_P fwd SpeI	ACTAGTAATTCTTGAAGACGAAAGGG
CymR_P oe rev	ACTCTTCCTTTTTCAATCTT
CymR gen fwd oe	AACATTGAAAAAGGAAGAGTATGAGTCCAAAGAGAAGAAC
CymR gen T7tt rev 1	CCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTTGCTAGCGCTTGAATTTCGCGTAC
CymR gen T7tt rev 2 SpeI	ACTAGTCTAGCATAACCCCTTGGGGCCTCTAAACG

LacI_SpeI_fwd	CAACTAGTGTTCTTTCCTGCGTTATCCCC
LacI_SpeI_rev	CAACTAGTACGCCAGAAGCATTGGTG

Table S2: Summary of cumate-induced eGFP expression and growth after 24 h based on *R. eutropha* H16 (pKRC-P_{j5}-eGFP) and of IPTG-induced eGFP expression and growth after 24 h based on *R. eutropha* RS1 (pKRL-P_{j5}-eGFP)

***R. eutropha* H16**	**Inducer p-cumate**	**OD_{600}**	**RFU**	***R. eutropha* RS1**	**Inducer IPTG**	**OD_{600}**	**RFU**
pKRSF1010Δegfp		19,5	0	pKRSF1010Δegfp		20,8	0
pKRC-P_{j5}-eGFP	uninduced	19,7	640	pKRL-Pj5-eGFP	uninduced	17,1	5400
pKRC-P_{j5}-eGFP	30 µM	19,7	19400	pKRL-Pj5-eGFP	0,01 mM	9,7	20400
pKRC-P_{j5}-eGFP	60 µM	19,5	20700	pKRL-Pj5-eGFP	0,1 mM	7,4	33100
pKRC-P_{j5}-eGFP	122 µM	19,7	21200	pKRL-Pj5-eGFP	1 mM	7,0	36100

Design of integration vector

The vector pInt_lacY_phaC was constructed by combining the pK470 backbone with the mobilization region of the RP4 plasmid and the phaC homologous regions. The MOB region was introduced by two PCR reactions with the primer pair's pK470_fwd_SpeI/ pK470_rev_PstI for the backbone (template pK470) and MOB_oriT_RP4_Fwd/ MOB_oriT_RP4_rev for the MOB region (template RP4). The fragments were restricted with *Spe*I and *Pst*I and via ligation the intermediate plasmid pK470 MobRP4 was created. The phaC1_1 and phaC1_2 regions were introduced by amplifying both regions out of genomic DNA of *R. eutropha* H16 with phaC1_1 Fwd_ SpeI and phaC1_1 Rev SpeI and phaC1_2_Fwd Xho/ phaC1_2_Rev_XhoI. The homologous regions were added subsequently by digesting pK470_MOBRP4 with *Spe*I and ligate with phaC1_1 restircted with *Spe*I. Afterwards the phaC1_2 region was added by digesting pK470_MOBRP4 with *Xho*I and ligate it with phaC1_2 also restricted with *Xho*I. The expression cassete constisting of the *lacY* gene (JF300162.1) under the control of the constitutive H16_B1772 promoter (described in Gruber et al.) was obtainend by amplifiing the promotor out of genomic DNA of *R. eutropha* H16 by PCR with H16_1772 SphI Fwd/ H16_1772 rev primers. The *lacY* gene was amplified by PCR out of genomic DNA from *E. coli* MG1655 with lacY fwd/lacY rev. The H16_B1772 fragment contained an overhang of 24 bp with the lacY fragment and therfore both fragments were combined by an overlap extension PCR. The whole casset was subsequently restricted and cloned into pK470_MOBRP4 amplified by phaC1 Rev SpeI SphI,HindIII_rrnB and restricted with the correspoding enzymes. The final plasmid pInt_lacY_phaC_loxP was constructed via two PCR products. The first product, the backbone of the vector, containing the 3' loxP, phaC1, lacY and the ColE1, was amplified by two subsequent PCR reactions with the plasmid pInt_lacY_phaC as a template. The first reaction was performed with phaC1_2_fwd_loxP_XmaI and ter_kanR_rev_StuI/AvrII/SpeI and used as template for the second PCR with phaC1_2_fwd_loxP_XmaI and Int_KanR_rev. The second fragment, containing the 5' loxP site and the CmR, was amplified with CmR_fwd_StuI_loxP and CmR_rev_XmaI as primers. The gained fragments were restricted with *Xma*I and *Stu*I and ligated resulting in the vector pInt_lacY_phaC_loxP. The vector pCM470_MOBRP4 was constructed to serve as a template for subseqeunt cloning steps. The backbone of pK470_MOBRP4 was amplified by PCR with colE1 pK470 Fwd/rrnB pK470 Rev and the CmR with Fwd cmR NotI/Rev cmR SpeI using the plasmid pSA as template Both PCR products were phosphorylated and ligated. After this exchange of the resistance marker the plasmid pCM-Cre was constructed by combining two fragments. For the first fragment the backbone of pCM470_MobRP4 containing the rrnB terminator, the Cm^r, the origin colE1 and the mobilization region of the RP4 plasmid was amplified with Rrnb-overlap-Cre+SmiI-fwd, MobRP4-overlap-CymR+SmiI-rev primers including a *Smi*I restriction site at

the end. Afterwards the gained fragment was digested with *Smi*I. The second fragment consists of three different sequences which were aligned via overlap extension PCR. The *cre* recombinase was amplified out of genomic DNA of bacteriophage P1 using Cre-overlap-Ptac-CymO-fwd, Cre-overlap-rrnb+SmiI-rev. The tac promoter, including the cumate operator was amplified using PTac-CymO-1/Ptac-Cymo-overlap-Cre-rev primers and pK470 as template and combined with Cre with a subsequent PCR performed with PTac-CymO-2 and Cre-overlap-rrnb+SmiI-rev. The third fragment, the cymate repressor was amplified using CymR-overlap-MobRP4+SmiI-fwd, CymR-overlap-Ptac-rev using pKRC-P_{J5}-egfp as template. All two fragments were used as template (equal molar ratios) for an overlap extension PCR with CymR-overlap-MobRP4+SmiI-fwd, Cre-overlap-rrnb+SmiI-rev primers including *Smi*I restriction sites at the end, afterwards the gained fragment was cut with *Smi*I and ligated with the corresponding fragment one, which was also previously digested with *Smi*I.

Chapter 4

3.4 CbbR and RegA regulate *cbb* operon transcription in *Ralstonia eutropha* H16

Contribution to this Chapter

Planning experiments ~ 95%

Laboratory work ~ 100%

Writing manuscript ~ 90%

Submitted to the Microbiology Journal

Editing and additional suggestions were provided by Dr. Petra Heidinger and Prof. Helmut Schwab

CbbR and RegA regulate *cbb* operon transcription in *Ralstonia eutropha* H16

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Abstract

The Gram-negative β-proteobacterium *Ralstonia eutropha* H16 is able to grow lithoautotrophically by utilizing CO_2 and H_2 as sole carbon and energy sources, respectively. CO_2 is fixed by the CBB cycle, which is encoded in duplicate on the genome of *R. eutropha* H16. The transcription of both *cbb* operons is controlled by the transcription regulator CbbR dependent on intracellular PEP levels as a response to the carbon-state of the cell. As demonstrated in this study transcription control of both *cbb* operons appears to be more complex and additionally involves, next to CbbR, the transcription regulator RegA as part of the global transcription regulation system RegA/RegB. The identification of a highly conserved RegA/RegB homologue in *R. eutropha* H16 and experimental evidence gathered in this study reveal that RegA plays a crucial role in the transcription control of both *cbb* promoters. RegA is able to induce *cbb* promoter activity and controls transcription in combination with CbbR dependent on cellular PEP concentrations. These results clearly demonstrate that RegA plays an important role in *cbb* operon transcription regulation and may also be relevant for the control of other energy-utilizing and energy-generating pathways of *R. eutropha* H16.

Keywords: *Ralstonia eutropha* H16; CO_2 uptake; transcription control; *cbb* operon; RegA/RegB

1. Introduction

The facultative chemolithoautotrophic Gram-negative bacterium *Ralstonia eutropha* H16 (now named *Cupriavidus necator* H16) is a soil-dwelling prokaryote that possesses a multi replicon genome consisting of two chromosomes and a megaplasmid (pHG1) (1). While genetic information regarding housekeeping genes, central carbon and energy metabolism is encoded chromosomally, the megaplasmid codes for a number of additional diverse metabolic pathways (1, 2). This also includes genetic information that is essential for the growth of *R. eutropha* H16 under lithoautotrophic conditions, which enables the utilization of CO_2 and H_2 as sole carbon and energy sources, respectively (3, 4).

The use of hydrogen as an energy source under lithoautotrophic growth conditions is accomplished by three types of hydrogenases that are involved in the oxidation of hydrogen. These hydrogenases are encoded by the *hox* operon located on pHG1 and belong to the family of [NiFe]-hydrogenases, including a membrane-bound hydrogenase (MBH), a soluble hydrogenase (SH) and a regulatory hydrogenase (RH) (5). Molecular hydrogen is detected by RH, which subsequently activates the transcription of MBH and SH genes with the help of the associated sensor kinase HoxJ and the transcription regulator HoxA (5, 6). The membrane associated MBH oxidizes H_2 in order to feed electrons into the electron transport chain driving ATP generation and the cytosolic SH oxidizes H_2 by reducing NAD+ to NADH (7). Due to the O_2 and CO tolerance of [NiFe]-hydrogenases hydrogen oxidation can additionally serve as an energy source under a variety of growth conditions (8, 9). The main carbon source for *R. eutropha* H16 under lithoautotrophic growth conditions is carbon dioxide, which is assimilated by the enzymes of the Calvin-Benson-Bassham (CBB) cycle. CO_2 fixation itself is carried out by the key enzyme ribulose-1,5-bisphosphate-carboxylase/-oxygenase (RuBisCO) type I, which enables the carboxylation of ribulose-1,5-bisphosphate (RuBP). The unstable product of this reaction immediately hydrolyses into two molecules of 3-phosphoglycerate, which are further reduced to glycerinaldehyde-3-phosphate. The triose phosphates are then used in the multistep regeneration part of the CBB cycle to yield ribulose-5-phosphate, which is phosphorylated in a final step to RuBP by phosphoribulokinase PRK/CbbP (4, 10). However, before RuBP can bind to RuBisCO the enzyme must bind Mg^{2+} as cofactor and needs to be carbamylated by the non-substrate CO_2 in order to be catalytically active. Premature binding of RuBP to non-carbamylated RuBisCO inactivates the enzyme. Consequently, the functionality of the entire CBB cycle depends on the presence of active RuBisCO and particularly CbbX, a RuBisCO activase (11, 12). Structural and functional studies of a CbbX homologue identified in *Rhodobacter sphaeroides* revealed that this AAA+ ATPase type protein forms a hexameric ring that interacts with RuBisCO in order to facilitate the release of inhibitory RuBP from RuBisCO, counteracting an inactivation of RuBisCO and the CBB cycle (13). The importance of CbbX activity is further supported by the observation of

impaired autotrophic growth in *cbbX* knock-out strains of *R. eutropha* H16 and *R. sphaeroides* (4, 14). Another enzyme that was found to play an important role in maintaining RuBisCO activity is CbbY, a xylulose-1,5-bisphosphate (XuBP) phosphatase (15). CbbY converts XuBP, a strong inhibitor of RuBisCO activity, to xylulose-5-phosphate, which in turn is converted to RuBP. XuBP is initially formed in small amounts by a RuBisCO side activity next to the carboxylation of RuBP (15–17). However, RuBisCO inactivation by XuBP appears to be less severe, as *R. eutropha* H16 and *R. sphaeroides cbbY* knock-out strains showed decreased RuBisCO activity but were not affected in autotrophic growth unlike comparable *cbbX* knock-out strains (4, 14).

The enzymes of the CBB cycle are encoded in the genome of *R. eutropha* H16 in duplicate; one operon is located on chromosome two and an almost identical copy on the megaplasmid. In comparison to the chromosomal *cbb* operon, the operon located on pHG1 lacks the gene coding for CbbB, a formate dehydrogenase like protein (Figure 1) (1, 4). Nevertheless a high degree of homology is shared by the *cbb* operons including the transcriptional regulation mechanism. In either case transcription of the entire operon is driven by a single σ^{70} promoter (P_{cbb}) located directly upstream of $cbbL_{C,P}$; no alternative internal transcription starts were identified so far (4, 18, 19). However, the relative abundance of *cbb* gene transcripts is affected by premature transcription termination induced by the formation of an mRNA based stem-loop in the intergenic region of *cbbS* and *cbbX*, which causes different gene expression levels within the *cbb* operons (19). Nonetheless, the main regulation of *cbb* operon transcription is executed by the LysR-type transcriptional regulator (LTTR) CbbR that directly controls the activity of P_{cbb}. The gene coding for CbbR is located upstream of the chromosomal operon and is under the control of a weak constitutive promoter. A very similar arrangement is also present on pHG1. However, no complete open reading frame is located upstream of the pHG1 *cbb* operon that could result in an active CbbR product (4, 18).

LTTRs like CbbR commonly act as a repressor or activator for a target gene or operon. This type of transcription regulator typically consists of an N-terminal DNA binding domain with a helix-turn-helix motif linked to a C-terminal regulatory domain that includes effector binding sites and domains involved in oligomerization (20, 21). LTTRs usually possess DNA binding sites in the proximity of the target promoter, which are defined as the activator binding site and regulator binding site; the regulator binding site is typically located upstream of the promoter whereas the activator binding site may overlap with the promoter sequence. In many cases additional DNA binding sites were identified and varying binding affinities of LTTRs to each binding site were observed (20–22). LTTRs bind in a multimeric form to DNA in order to regulate gene transcription by DNA bending; so far the formation of LTTR dimers, tetramers as well as octamers was verified (23–25). The state of

multimerization and the binding affinity of LTTRs to the particular DNA binding sites are considerably influenced by the presence of effector molecules; these signal molecules often represent a direct cellular metabolic feedback control (21). Altogether, this establishes a mechanism that regulates the transcription of target genes by bending DNA and controlling protein-protein interactions with the alpha subunit effector domains of the RNA polymerase at the target promoter (26).

In *R. eutropha* H16 CbbR regulates the transcription of both *cbb* operons by binding as a tetramer to an activator and a regulator binding site in the vicinity of the *cbb* promoter. Phosphoenolpyruvate (PEP) was identified as a signal metabolite for CbbR significantly repressing transcription of both *cbb* operons with increasing concentrations. The transcription regulation influenced by PEP, which is central to the carbon metabolism of the cell, is thought to reflect a feedback control depending on the carbon-state of the cell (4, 27). However, a more elaborate regulation mechanism that additionally implies a cellular energy-state dependent feedback control is expected to be involved in the transcription of both *cbb* operons in order to control the energy demanding process of carbon dioxide fixation.

In *R. sphaeroides* and *Rhodobacter capsulatus* transcription of *cbb* operons is also controlled by CbbR and a signal metabolite reflecting the carbon-state of the cell (28). However, a global transcription regulation system composed of a membrane associated histidine sensor kinase (RegB) and a transcription regulator (RegA) additionally influences the transcription of *cbb* operons (29–31). RegA~P, phosphorylated by RegB, binds as a dimer to defined DNA binding domains located upstream of the *cbb* operon and interacts with CbbR to regulate transcription in response to the redox state of the cell. The regulation of RegB kinase activity, and consequently the phosphorylation state of the transcription regulator RegA, is significantly inhibited under aerobic compared to anaerobic conditions (30). Inactivation of RegB activity is based on the sulfonation of its free thiol groups, binding of oxidized ubiquinone and the formation of inactive RegB tetramers in the presence of higher oxygen concentrations (32, 33). Next to the involvement in the regulation of carbon fixation in *R. sphaeroides* and *R. capsulatus*, the RegA/RegB system was identified to act as a global transcription regulation system that participates in the control of nitrogen fixation, respiration, electron transport, hydrogen oxidation and heme biosynthesis (29, 30). Highly conserved homologues of the RegA/RegB system were identified in numerous proteobacteria and were found to be similarly involved in the control of energy-utilizing and energy-generating processes responding to environmental and cellular redox state (30). Homologues of RegA and RegB including functionally relevant and conserved motives such as a RegB ubiquinone binding pocket, redox sensitive cysteine residues or the phosphorylation site of RegA can also be found in *R. eutropha*

H16 (Figure 2 and S1). However, neither the activity nor the functionality of the RegA/RegB system in *R. eutropha* H16 was described until now.

In this study experimental proof is presented that provides evidence for the involvement of the global transcription regulator RegA in the control of *cbb* operon transcription in *R. eutropha* H16. It could be demonstrated *in vivo* that RegA is capable to induce activity of both *cbb* promoters in *R. eutropha* H16 in addition to the transcription regulation executed by CbbR. Moreover, a significant difference in transcription regulation of the pHG1 and chromosome encoded *cbb* promoters with respect to RegA dependent regulation could be revealed. Altogether, this study provides strong evidence for a transcription regulation of both *cbb* operons based on CbbR and RegA as well as an active role of the global transcription regulation system RegA/RegB in *R. eutropha* H16.

2. Materials and Methods

2.1 Strains, plasmids and primers

All strains and plasmids used in this study are listed in tables 1 and 2. Primers used for PCR amplifications are summarized in table S1.

2.2 Cultivation of *E. coli* strains

E. coli TOP10 and *E. coli* XL1 cells were cultivated at 37°C on lysogeny broth (LB) media with ampicillin [100 µg/ml], chloramphenicol [25 µg/ml] or kanamycin [40 µg/ml] according to application. Succinate or glucose were supplied as carbon sources at 0.3% w/v in 96 deep well plates (DWP) cultivations and 1% w/v in shaking flask cultivations. Depending on the carbon source, low cellular PEP levels of approximately 0.09 mM are obtained in *E. coli* cells cultivated in liquid LB media supplemented with glucose and high PEP levels of approximately 0.96 mM in *E. coli* cells grown in liquid LB media supplemented with succinate (27, 34). In order to screen for promoter activity, LB agar plates were supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) [20 µg/ml]. All basic media components and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), Carl Roth (Arlesheim, Germany) and Becton Dickinson and Company (Franklin Lakes, NJ, USA).

2.3 DNA preparation and manipulation

Standard procedures for PCR, DNA preparation and manipulation as well as genomic DNA isolation were applied (35). Restriction enzymes and GeneJET Plasmid Miniprep Kits by Thermo Scientific (Waltham, MA, USA), Q5 High-Fidelity Polymerase by New England Biolabs (Ipswich, MA, USA), T4 DNA Ligation reaction mixtures, Wizard® SV Gel and PCR Clean-Up System by Promega (Madison, WI, USA) were used according to the manufacturers' protocols.

2.4 Plasmid construction

All plasmid backbones were assembled on the basis of pCC-1 (Figure S2). pCC-1 was created by combining PCR products encoding a colE1 *oriV* sequence and a chloramphenicol resistance gene (Cm^r) via *Spe*I and *Not*I. The colE1 *oriV* sequence was amplified with primers colE1-SpeI-fwd and

colE1-NotI-fwd from pMS470, the chloramphenicol resistance gene was amplified with primers CmR-SpeI-rev and CmR-NotI-rev from the pSa plasmid. Subsequently, PCR products encoding a 125 bp short or a 770 bp long region upstream of $cbbL_{C,P}$ (*H16_B1395* and *H16_PHG427)* including the *cbb* promoters encoded on the chromosome and pHG1, respectively (Figures 1 and S3), the reporter gene *lacZ* and a kanamycin resistance gene (Km^r) were assembled in an overlap extension PCR step to yield one DNA fragment containing Km^r, *lacZ* and the particular *cbb* promoter sequence. The DNA fragment encoding Km^r was amplified with primers rrnb-oe-KanR and KanR-SpeI-rev from pET28, lacZ was amplified with primers lacZ-OE-fwd and lacZ-OE-rev from pRS415 and the particular *cbb* promoter sequences were obtained from the genomic DNA of *R. eutropha* H16 using primers listed in Table S1. The plasmids encoding a region covering 790 bp upstream of *cbbR* (*H16_B1396*), which contains the *cbbR* promoter, were constructed in the same manner as the PCR products containing the *cbb* promoters. pCC-1 and the particular DNA fragments assembled by overlap extension PCR were digested with *Not*I and *Spe*I. The digested DNA fragments were ligated to create the plasmids pCK-A1 through pCK-C4 listed in Table 2. This step involved the replacement of Cm^r by one DNA fragment containing Km^r, *lacZ* and the particular *cbb* or *cbbr* promoter sequence. Moreover, the start codon of *cbbR* that is partially encoded on the complementary DNA strand of the 770 bp long chromosome derived *cbb* control region was deleted, since the partial *cbbR* gene resulted in an active, truncated CbbR product. Co-expression cassettes containing *regA* (*H16_A0202*) and *cbbR* (*H16_B1396*) were constructed by overlap extension PCR of DNA fragments coding for the constitutive promoter of the chloramphenicol resistance marker P_{Cmr}, *regA* or *cbbR* and a T7 terminator sequence. P_{Cmr} was amplified with primers PcmR-SpeI-fwd and PcmR-oe-rev from the pSa plasmid, *regA* and *cbbR* were amplified from the genomic DNA of *R. eutropha* H16 with primers RegA-fwd, RegA-rev, cbbRSD-fwd-XbaI and cbbR-rev-HindIII. The T7 terminator sequence was encoded on the primers. Depending on application the co-expression cassettes were cloned into the particular plasmids via *Not*I or *Spe*I restriction sites. *E. coli* TOP10 or *E. coli* XL1 cells were transformed with desired plasmids using standard electroporation protocols (35).

2.5 Promoter activity (Miller Unit) measurements

Overnight cultures (ONCs) of *E. coli* XL1 strains containing ß-galactosidase-based reporter plasmids were grown in liquid LB kanamycin [40 µg/ml] media overnight in 96 deep well plates (DWP plates) or shaking flasks according to application. The ONCs were used to inoculate LB media to an OD_{600} of 0.2 and cultures were grown in LB succinate or glucose media to a final OD_{600} of approximately 1.5. At this point 150 µl of the culture were added to 1 ml of Z-buffer pH 7 containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1mM $MgSO_4$ and 50 mM ß-mercaptoethanol.

Subsequently, 50 µl of chloroform as well as 25 µl 0.1% SDS were added and the samples were mixed by pipetting, followed by incubation at room temperature for 30 minutes to allow phase separation. Afterwards 125 µl of the aqueous phase of each sample were transferred to a 96 well microtiter plate. 25 µl of a [4 mg/ml] substrate solution of 2-nitrophenyl-β-D-galactopyranoside (ONPG) dissolved in sodium phosphate buffer (0.1 M, pH 7) were added to each well and the samples were incubated at room temperature for 5 or 10 minutes according to the intensity of coloration. The reactions were stopped by adding 65 µl of a 1 M Na_2CO_3 solution. Absorbance values of each sample were determined at 420 nm and 550 nm with FLUOstar Omega (BMG Labtech, Ortenberg, Germany). OD_{600} values of each culture were determined with respect to appropriate dilutions in 96 well microtiter plates using FLUOstar Omega (BMG Labtech, Ortenberg, Germany). Lastly, the promoter activity was determined in Miller Units (36). *E. coli* XL1 cells served as negative control.

3. Results

3.1 CbbR transcription regulation of pHG1 and chromosome encoded *cbb* promoters

In order to study *cbb* promoter transcription regulation the heterologous host *E. coli* XL1 and a set of plasmids encoding *lacZ* as a reporter gene were employed. In these reporter constructs a 125 bp short or a 770 bp long control region of each *cbb* operon was cloned directly upstream of *lacZ* in order to compare *cbb* promoters derived from pHG1 and chromosome two of *R. eutropha* H16 (Table 2). In addition, CbbR, RegA or both transcription regulators were co-expressed using a weak, constitutive promoter (P_{Cmr}) to evaluate the effect on *cbb* promoter activity. These *E. coli* XL1 based strains were cultivated in the presence of low or high levels of the signal metabolite PEP, which is known to affect a feedback response mediated by CbbR (4). Therefore, *E. coli* XL1 cells carrying the designed plasmids were grown in liquid LB media supplemented with glucose to yield low cellular PEP levels of approximately 0.09 mM and in liquid LB media supplemented with succinate to obtain high PEP levels of 0.96 mM (34)(27). The actual *cbb* promoter activity was characterized on the basis of different plasmid vectors, which were used to determine ß-galactosidase activity defined in Miller Units (MU). The obtained results are summarized in Figures 3 and 4.

E. coli XL1 strains carrying plasmids pCK-A1, pCK-A2, pCK-B1 and pCK-B2 that do not co-express CbbR or RegA were used to determine the basal activity of the chromosomal and pHG1 *cbb* promoters. The short and long *cbb* promoter regions derived from pHG1 exhibited a weak constitutive activity in the range of 50 MU, which was maintained in the presence of low or high PEP

concentrations (Figure 3). On the contrary, the basal promoter activity for the short and long chromosomal *cbb* promoter regions was determined to be in the range of 220 MU under low and high PEP concentrations (Figure 4). The MU values obtained for *E. coli* XL1 strains carrying plasmids pCK-A1, pCK-A2, pCK-B1 and pCK-B2 that do not co-express a transcription regulator are almost identical for the particular *cbb* promoters irrespectively of low or high cellular PEP concentrations applied. On the other hand, the formation of β-galactosidase was highly responsive to cellular PEP levels in *E. coli* XL1 strains carrying plasmids pCK-A3, pCK-A4, pCK-B3 and pCK-B4, which co-express only CbbR. *E. coli* XL1 strains carrying plasmids pCK-A3 and pCK-A4 encoding the short and long chromosomal P_{cbb}, respectively, were grown in the presence of low cellular PEP concentrations, which resulted in an increase of MU values by a factor of 16 compared to the basal promoter activity of the chromosome encoded *cbb* promoter (Figure 4). The promoter activities exhibited by the same *E. coli* XL1 strains cultivated in the presence of high cellular PEP concentrations were increased approximately 3,5 times in comparison to the basal activity of the chromosomal *cbb* promoter. *E. coli* XL1 (pCK-B3) and *E. coli* XL1 (pCK-B4) encoding the short and long pHG1 *cbb* promoter regions exhibited an increase by a factor of 20 in MU values when grown in the presence of low cellular PEP levels and a sevenfold increase grown at high cellular PEP levels in relation to the basal promoter activity of the pHG1 encoded P_{cbb}.

3.2 Influence of RegA on *cbb* and *cbbR* promoter activity

A homologue of the global transcription regulation system RegA/RegB is encoded in *R. eutropha* H16 on chromosome one by genes *H16_A0202* and *H16_A0203*. The RegA/RegB system consists of the transcription regulator RegA and the membrane bound sensor histidine kinase RegB. The role of RegA and RegB was thoroughly studied in *R. sphaeroides* and *R. capsulatus*, where they actively take part in the transcription regulation of numerous energy-utilizing or energy-generating processes (29, 30). An alignment of RegA or RegB amino acid sequences derived from *R. sphaeroides*, *R. capsulatus* and *R. eutropha* H16 reveals that numerous conserved essential features of this system are also present in *R. eutropha* H16 (Figure 2 and Figure S1). This includes motives like the ubiquinone binding pocket, the phosphorylation site or the redox-active cysteine of the sensor kinase RegB and features such as the DNA binding helix-turn-helix motif and the site of phosphorylation of the transcription regulator RegA (Figure 2 and Figure S1). In order to evaluate a potential role of RegA in the regulation of *cbb* operon and *cbbR* transcription in *R. eutropha* H16, the transcription regulator RegA (*H16_A0202*) derived from *R. eutropha* H16 was co-expressed on plasmids pCK-A5, pCK-A6, pCK-A7, pCK-A8, pCK-B5, pCK-B6, pCK-B7, pCK-B8, pCK-C3 and pCK-C4 in *E. coli* XL1. Usually, the phosphorylation of RegA by the sensor kinase RegB is necessary for the formation of

RegA dimers, which is the predominantly active conformation of this transcription regulator (37). As previously reported, however, unspecific phosphorylation of RegA most likely occurs during expression in *E. coli* cells and enables the formation of RegA dimers (37, 38). For this reason RegB was not co-expressed.

The co-expression of the transcription regulator RegA on plasmids pCK-A5, pCK-A6, pCK-B5 and pCK-B6 in *E. coli* XL1 cells induced *cbb* promoter activities significantly (Figure 3 and 4). *E. coli* XL1 (pCK-A5) encoding the short chromosomal *cbb* promoter region exhibited MU values that were increased by a factor of approximately 24 when grown in the presence of low PEP concentrations and an increase of MU values by 27 times when grown in the presence of high PEP concentrations in relation to the basal activity of the chromosome encoded *cbb* promoter. The promoter activities for the long chromosomal *cbb* promoter determined on the basis of *E. coli* XL1 (pCK-A6) were increased by a factor of 12 under high PEP levels and a factor 11 under low PEP levels related to the particular basal *cbb* promoter activity. In comparison, *E. coli* XL1 (pCK-B5) encoding the short pHG1 *cbb* promoter region exhibited 140 and 160 fold increased MU values when grown under low and high cellular PEP levels related to the basal activity of pHG1 P_{cbb}. The MU values obtained for the long *cbb* pHG1 promoter region based on *E. coli* XL1 (pCK-B6) were increased approximately 100 and 110 fold under low and high PEP levels, respectively, compared to the basal pHG1 *cbb* promoter activity.

3.3 Influence of CbbR and RegA on *cbb* and *cbbR* promoter activity

Simultaneous co-expression of RegA and CbbR under the control of two identical, constitutive promoters resulted in significant induction of *lacZ* transcription in all constructs (Figure 3 and 4). The MU values for *E. coli* XL1 (pCK-A7) and *E. coli* XL1 (pCK-A8) encoding the short and long chromosomal P_{cbb} were increased by a factor of 33 and 38, respectively, under low PEP concentrations compared to the basal activity of the chromosomal *cbb* promoter. The promoter activities of the same strains grown under conditions of high PEP levels were increased only 17 times. *E. coli* XL1 (pCK-B7) and *E. coli* XL1 (pCK-B8) encoding the short and long pHG1 P_{cbb} when grown under conditions of low cellular PEP levels exhibited an increase in promoter activities by 160 and 170 fold, respectively, regarding the basal activity of pHG1 P_{cbb}. When the same strains were grown at high cellular PEP levels the activity was determined to be 70 times higher for the short and long control region in comparison to basal activity of pHG1 P_{cbb}. All *E. coli* XL1 strains co-expressing CbbR and RegA exhibited P_{cbb} activities that were influenced by cellular PEP concentrations. Similarly to the transcription regulation based on CbbR, the MU values were increased at a higher rate in the presence of low PEP levels and at a lower rate in the presence of high PEP levels. The

absolute MU values for *E. coli* XL1 strains carrying plasmids pCK-A7, pCK-A8, pCK-B7 and pCK-B8 were found to be in the same range in the presence of low or high PEP levels, respectively. Moreover, the MU values obtained for the combined co-expression of CbbR and RegA did clearly exceed the MU values determined for strains overexpressing only RegA or CbbR in the presence of low PEP levels. On the contrary, the promoter activity determined in the presence of high PEP levels was decreased below the values obtained for *E. coli* XL1 strains co-expressing only RegA under the same growth conditions, except for *E. coli* XL1 (pCK-A7) encoding the long chromosomal P_{cbb}. However, the absolute MU values were also found to be in the same range for *E. coli* XL1 strains carrying plasmids pCK-A7, pCK-A8, pCK-B7 and pCK-B8 in the presence of high PEP levels (Figure 3 and 4).

The activity of the *cbbR* promoter was characterized on the basis of plasmids pCK-C1, pCK-C2, pCK-C3 and pCK-C4, which either co-expressed CbbR and RegA together, CbbR or RegA alone or did not co-express a transcription regulator. However, due to the very low activity of the *cbbR* promoter absolute MU values were not reproducible on the basis of the ONPG assay. Nevertheless, the collected data clearly showed induced *cbbR* promoter activity at a very low level for *E. coli* XL1 (pCK-C3) and (pCK-C4) in the presence of co-expressed RegA. These results could be verified by X-Gal based LB agar plate assays, which enable a more sensitive detection of β-galactosidase activity compared to the ONPG assay (39). Again, a blue coloration of cultures was clearly visible only in the presence of co-expressed RegA for *E. coli* XL1 (pCK-C3) and (pCK-C4). An influence of the transcription regulator CbbR or the signal metabolite PEP on the activity of the *cbbR* promoter could not be observed.

4. Discussion

4.1 Comparison of pHG1 and chromosome encoded *cbb* operons

The *cbb* operons encoded on the genome of *R. eutropha* H16 share a high degree of homology on a nucleotide level, a similar number of genes and an identical arrangement of the particular *cbb* promoters as well as CbbR binding sites (Figure 1). However, the chromosome and pHG1 encoded *cbb* operons differ by two genes, which are only present on the chromosome. These genes code for a formate dehydrogenase like protein ($cbbB_C$) and the transcription regulator ($cbbR_C$) (Figure 1). A highly homologous DNA sequence similar to $cbbR_C$ is also located directly upstream of $cbbL_P$; however, no complete open reading frame is present that could result in the formation of a functional product (Figure 1 and S3). A comparison of the region covering approximately 700 bp upstream of the CbbR binding sites, a region that is likely to contain additional DNA binding sites of transcription regulators, reveals a significant degree of homology on a nucleotide level, but does also account for

numerous differences that appear to result from deletion events (Figure S3). On the contrary, the nucleotide sequence located directly upstream of *cbbL*$_C$ and *cbbL*$_P$ is almost identical and does only differ at five positions that are located in the *cbb* promoter region and the CbbR DNA binding sites (Figure 1).

4.2 Influence of CbbR on *cbb* promoter activity

The abovementioned variations occurring in the otherwise highly homologous nucleotide sequences of both *cbb* core promoters are likely responsible for the differences regarding the basal activity of the *cbb* promoters encoded on the chromosome and pHG1 (Figure 3 and 4). The nucleotide sequences of the *cbb* promoters differ at three positions in the CbbR binding site, at two positions in the *cbb* core promoter between the -35 and -10 box and at one position that is located between the *cbb* core promoter and the Shine-Dalgarno sequence (Figure 1). The differences occurring at positions outside of the *cbb* core promoter sequence are not located in regions that are reported to affect *cbb* promoter activity and therefore are not expected to influence the promoter's functionality (4, 18). However, Jeffke et al. (1999) showed that minor changes in the nucleotide sequence or variations in the length of the spacer sequence between the -35 and -10 box can influence the activity of the chromosomal *cbb* promoter significantly. Accordingly, the differences in the nucleotide sequence of the core promoter region are expected to cause the lower basal promoter activity observed for the pHG1 derived P_{cbb} when compared to the chromosome derived P_{cbb}. Furthermore, the basal activity of the particular *cbb* promoters was not influenced by varying levels of the signal metabolite PEP when CbbR was not co-expressed. This is in accordance to results of a previous study performed by Grzeszik et al., (2000).

However, Grzeszik et al., (2000) did also show that the activity of the chromosomally encoded *cbb* promoter is significantly influenced by varying cellular PEP concentrations in the presence of co-expressed CbbR, which also coincides with the data collected in this current study. In accordance with these findings, repressed *cbb* promoter activity was observed in cells grown in the presence of high cellular PEP levels and induction of P_{cbb} activity in cells grown at low cellular PEP levels (Figure 3 and 4). In addition to the data reported on the chromosomal *cbb* promoter by Grzeszik et al., (2000), the results obtained in this study clearly show that the *cbb* promoter located on pHG1 also responds to a transcription regulation based on CbbR and varying PEP levels. Furthermore, the activity of the chromosome and pHG1 encoded *cbb* promoters is increased by the same factor in comparison to the particular basal promoter activity when monitored under low or high PEP levels (Figures 3 and 4). Accordingly, the CbbR based transcription regulation exerts the same effect on

the chromosomal and pHG1 *cbb* promoters, the difference in absolute MU values results from the respective basal promoter activities.

4.3 Influence of RegA on *cbb* promoter activity

CbbR dependent transcription regulation of *cbb* operons can also be observed with *R. sphaeroides* and *R. capsulatus*. Moreover, transcription of these *cbb* operons was found to be influenced additionally by the RegA/RegB system, a global transcription regulation system that controls the majority of energy-utilizing and energy-generating processes in *R. sphaeroides* and *R. capsulatus* (29, 30). The identification of a RegA/RegB homologue in *R. eutropha* H16, which shares a high degree of conservation for all functionally relevant features on the amino acid level compared to the systems found in *R. sphaeroides* and *R. capsulatus* (Figures 2 and S1), points to a similar function of the RegA/RegB system in *R. eutropha* H16. In order to evaluate a potential influence of the RegA/RegB system on *cbb* operon transcription in *R. eutropha* H16, the effect of RegA on the activity of the chromosome and pHG1 encoded *cbb* promoters was assessed. It could be demonstrated that RegA is able to induce activity of both, the chromosome and pHG1 encoded *cbb* promoters (Figures 3 and 4), independent of cellular PEP levels and presence of CbbR. Moreover, RegA is able to significantly induce *cbb* promoter activity, which in the particular experimental set-up of this study exceeds the promoter activity levels induced solely by the action of CbbR. However, this might not reflect the natural situation in *R. eutropha* H16 as both transcription regulators were heterologously expressed from equally strong promoters. Nevertheless, the obtained data provides strong evidence that transcription of both *cbb* operons in *R. eutropha* H16 is additionally under the control of the RegA/RegB system. The collected results also reveal a considerable difference in promoter activity for the particular 125 bp short and 770 bp long control regions as well as a greater influence of RegA on the pHG1 *cbb* promoter compared to the chromosomal *cbb* promoter (Figures 3 and 4). So far the exact DNA binding motif, which is conserved only at a low level among RegA homologues (40, 41), and the number of putative DNA binding sites of RegA in the upstream region of both *cbb* operons are unknown for *R. eutropha* H16. However, at least one RegA DNA binding site should be located in the short chromosomal and pHG1 P_{cbb} control region promoting transcription in *E. coli* XL1 (pCK-A5) and (pCK-B5). The weaker influence of RegA on the short chromosomal compared to the short pHG1 P_{cbb} control region suggests a difference in DNA binding affinity likely determined by the differences observed in the corresponding nucleotide sequences (Figure 1).

The mode of transcription regulation executed by RegA could be similar to the one observed for a closely related homologue in *R. sphaeroides*, which induces DNA loop formation to control *cbb*

operon transcription (28). In accordance with these findings, the significantly reduced promoter activities observed in this study in the presence of co-expressed RegA for the long compared to the short *cbb* promoter regions could also result from the formation of a DNA loop. This RegA induced DNA loop could affect RNA polymerase recruitment and interactions with associated transcription regulators (42), causing a lower induction of *cbb* promoter activity for the long *cbb* control regions. This may support the assumption that a proper DNA loop cannot be established in case of the short *cbb* promoter control regions due to the short DNA sequence and lack of a sufficient number of RegA DNA binding sites. However, this could enable a direct access of RNA polymerase, unaffected by a DNA loop, and promote a stronger induction of promoter activity in the short *cbb* control regions. In comparison, co-expression of CbbR, which regulates transcription by DNA bending (4, 26), induces identical *cbb* promoter activities for the short and long control regions, respectively (Figures 3 and 4).

4.4 Influence of RegA and CbbR on *cbb* and *cbbR* promoter activity

The co-expression of CbbR and RegA clearly demonstrates that the activity of the *cbb* promoters derived from *R. eutropha* H16 is controlled by both transcription regulators in a combined manner. In all cases *cbb* promoter activity is induced by CbbR and RegA in a PEP dependent manner (Figures 3 and 4). As a consequence, *cbb* promoter activity is moderately induced under conditions of high PEP levels and strongly induced at low PEP levels. Moreover, *cbb* promoter activities obtained under low PEP levels exceeded the rate of induction observed for solely co-expressing CbbR or RegA in all experimental setups, thereby representing the highest rate of *cbb* promoter induction reported so far. On the contrary, the increase in *cbb* promoter activity was significantly lower in the presence of high PEP levels, which is most likely a result of the CbbR-based transcription regulation since RegA induced *lacZ* expression is not influenced by cellular PEP levels. Interestingly, despite the significantly different basal *cbb* promoter activities, the same influence of the CbbR-based transcription regulation on both *cbb* promoters and a stronger influence of RegA on the pHG1 compared to the chromosome encoded *cbb* promoter, all absolute MU values obtained under low and high PEP levels for the short and long chromosome or pHG1 derived *cbb* promoter constructs were almost identical. The sum of all effects generates an equally strong expression of the chromosome and pHG1 encoded *cbb* promoters with respect to this experimental setup presumably providing the same amounts of CbbR and RegA. However, this does most likely not resemble the natural situation in *R. eutropha* H16, which most likely differs for the amounts of CbbR or active RegA under different growth conditions. Nevertheless, these findings coincide with previous results

showing that *cbb* operon transcription in *R. eutropha* H16 appears to be equally strong from chromosome two and pHG1 (43).

Comparing the results of this study with the mechanism described to regulate transcription of the *cbb* operon in *R. sphaeroides* (29) it becomes apparent that a similar kind of regulation based on RegA and CbbR may control the transcription of *cbb* operons in *R. eutropha* H16. In both cases the transcription regulators RegA and CbbR are involved in the control of *cbb* operon transcription and in either case CbbR executes a carbon-dependent feedback mechanism. On the other hand, RegA as a part of the RegA/RegB system, represents a feedback control in response to the energy-state of the cell (44). In case of *R. sphaeroides*, transcription regulation of the *cbb* operon is influenced by DNA loop formation induced by RegA, which is formed on the basis of four RegA DNA binding sites that are situated up to 450 bp upstream of the *cbbL* promoter. One of these RegA DNA binding sites was found to overlap with the CbbR binding site promoting protein-protein interactions between RegA and CbbR (28, 45). In accordance with the obtained results in this study, similar aspects may also play a role in the regulation of *cbb* operon transcription in *R. eutropha* H16. However, the exact mechanism of *cbb* operon transcription regulation still needs to be studied in detail in *R. eutropha* H16. This includes the characterization of RegA DNA binding sites, putative protein-protein interactions between CbbR and RegA or the possibility of RegA induced DNA looping.

Finally, the activity of the *cbbR* promoter was also found to be positively influenced by RegA. Controlling *cbb* operon and *cbbR* transcription, the RegA/RegB system found in *R. eutropha* H16 seems to entirely influence CO_2 fixation on the level of transcription. The involvement of the RegA/RegB system in the transcription control of *cbbR* and *cbb* operons in *R. eutropha* H16 indicates a mode of operation for the RegA/RegB system similar to the homologues in *R. sphaeroides* or *R. capsulatus,* controlling the transcription of numerous energy-utilizing and energy-generating processes to maintain the cellular redox poise (32).

5. Authors' Contributions

SG, PH, HS planned and started the project. Wet laboratory work was carried out by SG. SG and PH wrote the manuscript. All authors read, corrected and approved the final version.

6. Acknowledgment

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Figure(s)

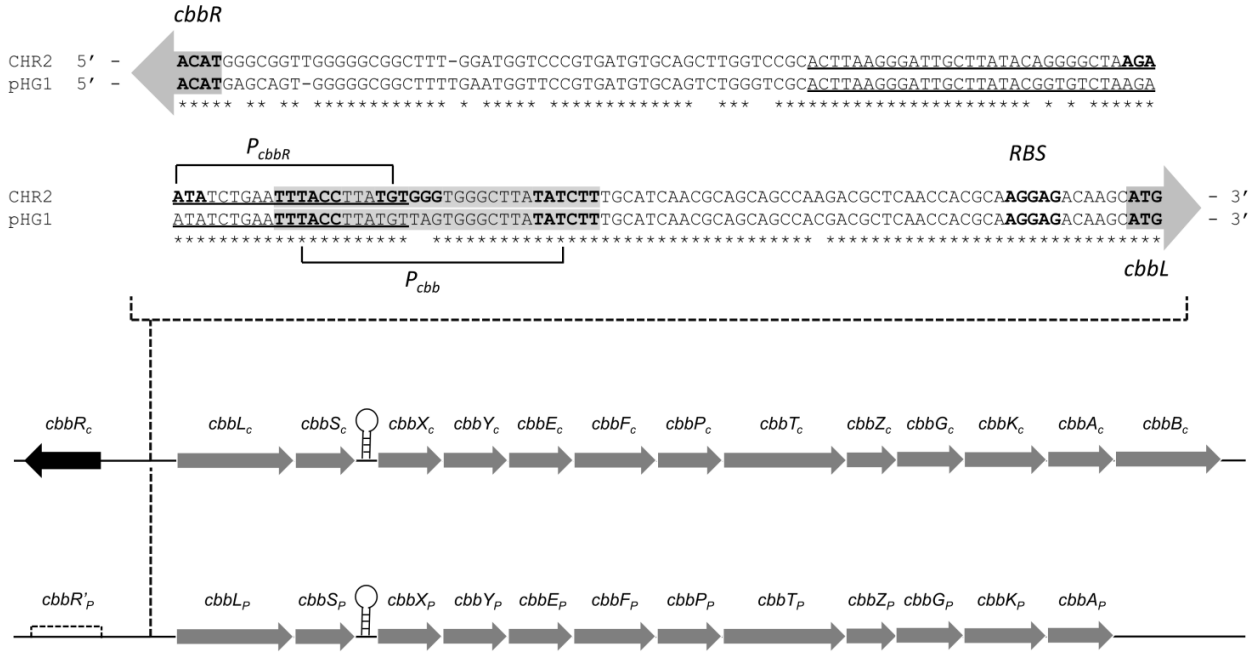

Figure 1: Schematic view of the *cbb* operons encoded on chromosome two and the megaplasmid of *R. eutropha* H16 including the structural *cbb* genes and the $cbbR_c$ gene coding for the transcription regulator CbbR. $CbbR'_p$ relates to the highly homologous region similar to CbbR found on pHG1. The regions located directly upstream of *cbbL* are shown in a sequence alignment, identical nucleotides are labelled with asterisks. The nucleotide sequences contain the CbbR DNA binding site (underlined), the *cbb* promoters (shaded), the *cbbR* promoter, the ribosome binding site (RBS), the mRNA-based stem-loop and the start codons of *cbbR* and *cbbL*. The figure was adapted from Bowien and Kusian (2002).

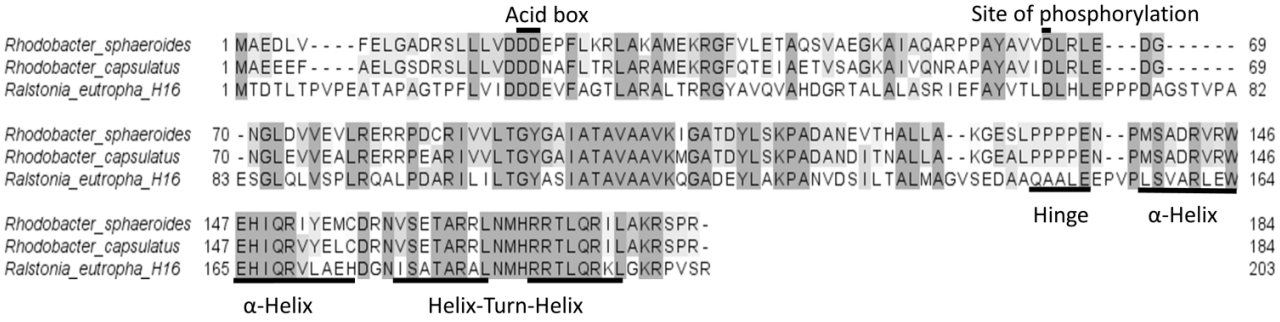

Figure 2: Sequence alignment of RegA amino acid sequences derived from *R. sphaeroides*, *R. capsulatus* and *R. eutropha* H16. The amino acid sequences of the transcription regulator RegA were aligned using ClustalW (46). The results were visualized with Jalview (47). Identical residues are shaded according to the degree of conservation in all amino acid sequences. The RegA sequence alignment reveals conserved acid boxes, the site of phosphorylation, a hinge region, α-helix and helix-turn-helix motives.

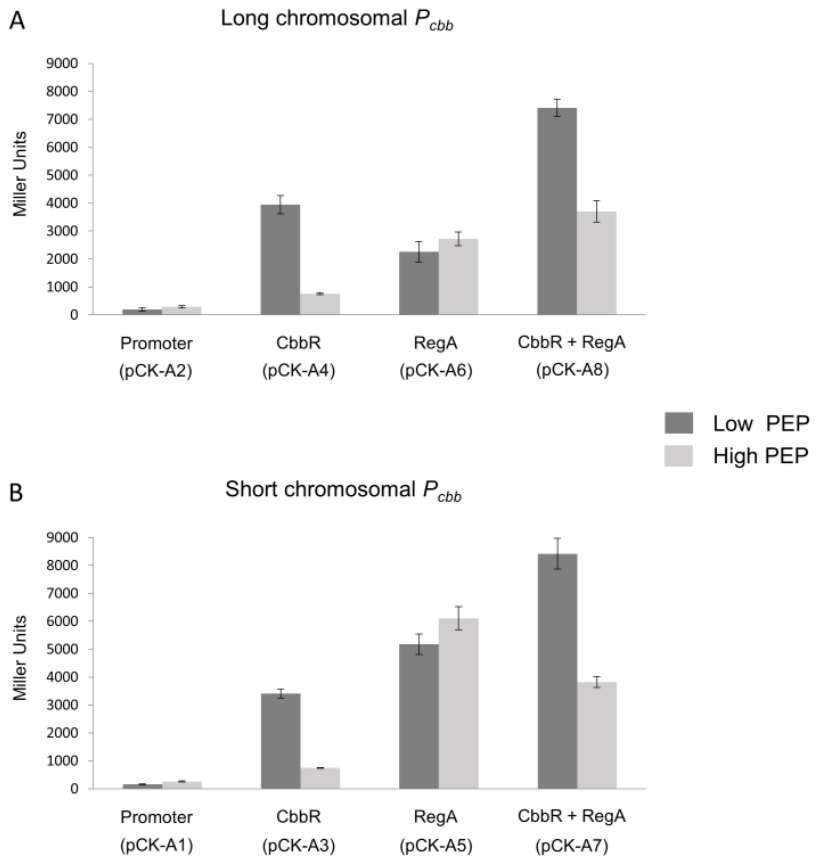

Figure 3: Activity of the chromosome encoded *cbb* promoter in Miller Unit values based on *E. coli* XL1 strains carrying plasmids that do not co-express a transcription regulator and plasmids that co-express the transcription regulators CbbR, RegA or both. The *cbb* promoter activity values for the *E. coli* XL1 strains grown in the presence of low PEP levels are indicated in dark grey, the *cbb* promoter activity values obtained under high PEP levels are shown in light grey. **A:** Miller Unit values for *E. coli* XL1 strains carrying plasmids that encode the long chromosomal *cbb* promoter region grown in the presence of low or high PEP levels. **B:** Miller Unit values for *E. coli* XL1 strains carrying plasmids that encode the short chromosomal P_{cbb} region grown in the presence of low or high PEP levels.

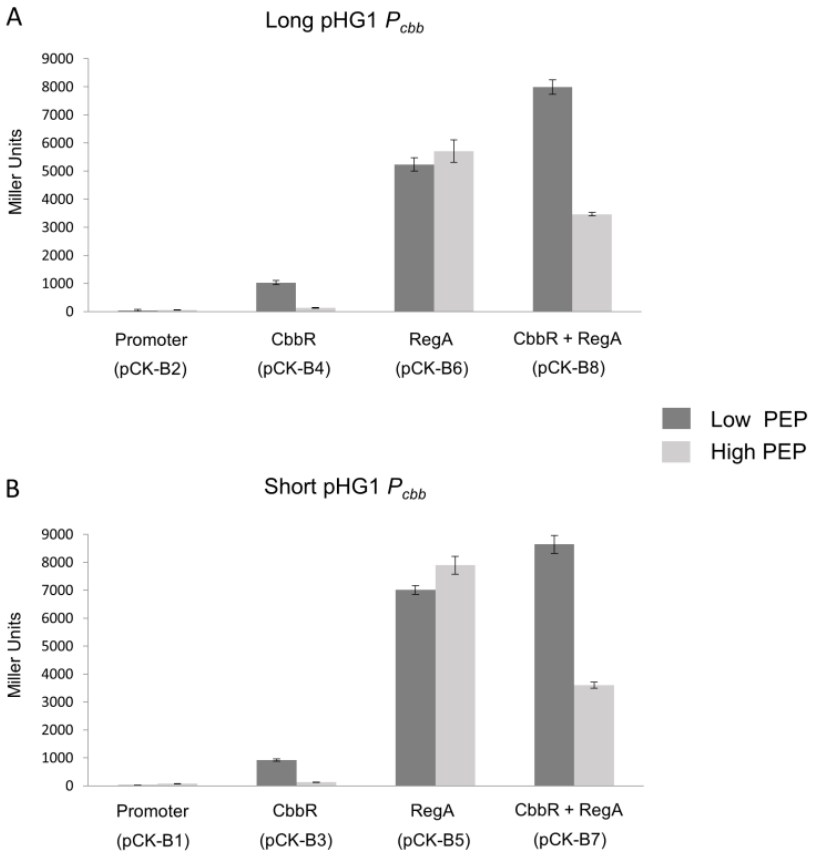

Figure 4: Activity of the pHG1 encoded *cbb* promoter activities in Miller Unit values based on *E. coli* XL1 strains carrying plasmids that do not co-express a transcription regulator and plasmids that co-express the transcription regulators CbbR, RegA or both. The *cbb* promoter activity values for the *E. coli* XL1 strains grown in the presence of low PEP levels are indicated in dark grey, the *cbb* promoter activity values obtained under high PEP levels are shown in light grey. **A:** Miller Unit values for *E. coli* XL1 strains carrying plasmids that encode the long pHG1 *cbb* promoter region grown in the presence of low or high PEP levels. **B:** Miller Unit values for *E. coli* XL1 strains carrying plasmids that encode the short pHG1 P_{cbb} region grown in the presence of low or high PEP levels.

Table1: Strains used in this study

Strain	Description	References or Source
E. coli XL1	F´ *::Tn10 proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Δ(lacZ)M15/ recA1 endA1 gyrA96* (Nal^{R}) *thi hsdR17 (rK*$^{-}$ *mK*$^{+}$*) glnV44 relA1 lac*	Stratagene
E. coli TOP10	F´(*proAB, lacIq*, *lacZ*ΔM15, Tn10(tet-r)), *mcrA*, Δ(mrr-hsdRMS-mcrBC), Φ80ΔlacZΔM15, Δ*lacX74*, *deoR*, *recA1*, *araD139*(*ara, leu*), 7697, *galU*, *galK*, λ-, *rpsL*(streptomycin-r), *endA1*, *nupG*	Invitrogen
R. eutropha H16	Wild-type, gentamicin resistant	DSMZ 428[a]

[a] DSMZ, Deutsche Sammlung für Mikroorganismen und Zellkulturen.

Table 2: Plasmids used in this study

pET28	Km^{r}, P_{T7}, *lacI*, f1 origin, pBR322 origin	Novagen
pMS470Δ8	Ap^{r}, P_{tac}, *lacI*, colE1 origin of replication	(48)
pRS415	Ap^{r}, *lacZ*, *lacA*, *lacY*, pUC origin of replication	(49)
pSa	broad-host-range plasmid, IncW, Cm^{r}	(50)
pCC-1	Cm^{r}, colE1 origin of replication	This study

pCK-A1	Km^r, short chromosomal P_{cbb} , *lacZ*, colE1 origin of replication	This study
pCK-A2	Km^r, long chromosomal P_{cbb} , *lacZ*, colE1 origin of replication	This study
pCK-A3	Km^r, short chromosomal P_{cbb} , *lacZ*, *cbbR*, colE1 origin of replication	This study
pCK-A4	Km^r, long chromosomal P_{cbb} , *lacZ*, *cbbR*, colE1 origin of replication	This study
pCK-A5	Km^r, short chromosomal P_{cbb} , *lacZ*, *regA*, colE1 origin of replication	This study
pCK-A6	Km^r, long chromosomal P_{cbb} , *lacZ*, *regA*, colE1 origin of replication	This study
pCK-A7	Km^r, short chromosomal P_{cbb} , *lacZ*, *cbbR*, *regA*, colE1 origin of replication	This study
pCK-A8	Km^r, long chromosomal P_{cbb} , *lacZ*, *cbbR*, *regA*, colE1 origin of replication	This study
pCK-B1	Km^r, short pHG1 P_{cbb} , *lacZ*, colE1 origin of replication	This study
pCK-B2	Km^r, long pHG1 P_{cbb} , *lacZ*, colE1 origin of replication	This study
pCK-B3	Km^r, short pHG1 P_{cbb} , *lacZ*, *cbbR*, colE1 origin of replication	This study
pCK-B4	Km^r, long pHG1 P_{cbb} , *lacZ*, *cbbR*, colE1 origin of replication	This study
pCK-B5	Km^r, short pHG1 P_{cbb} , *lacZ*, *regA*, colE1 origin of replication	This study
pCK-B6	Km^r, long pHG1 P_{cbb} , *lacZ*, *regA*, colE1 origin of replication	This study
pCK-B7	Km^r, short pHG1 P_{cbb} , *lacZ*, *cbbR*, *regA*, colE1 origin of replication	This study
pCK-B8	Km^r, long pHG1 P_{cbb} , *lacZ*, *cbbR*, *regA*, colE1 origin of replication	This study
pCK-C1	Km^r, P_{cbbR} , *lacZ*, colE1 origin of replication	This study
pCK-C2	Km^r, P_{cbbR} , *lacZ*, *cbbR*, colE1 origin of replication	This study

pCK-C3	Km^r, P_{cbbR} , *lacZ*, *regA*, colE1 origin of replication	This study
pCK-C4	Km^r, P_{cbbR} , *lacZ*, *cbbR*, *regA*, colE1 origin of replication	This study

Supplementary Data

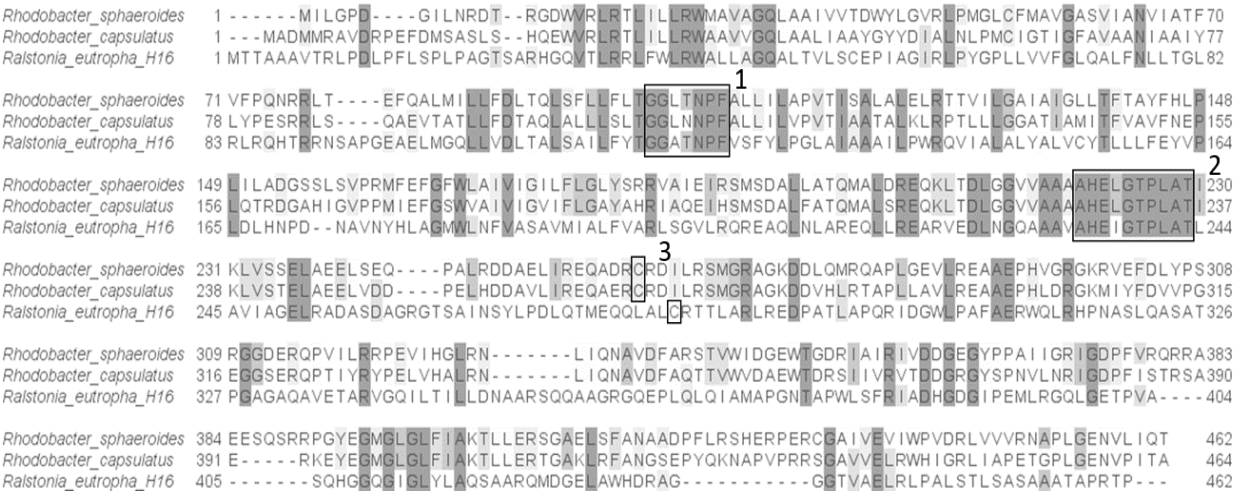

Figure S1: Sequence alignment of RegB amino acid sequences derived from *R. sphaeroides*, *R. capsulatus* and *R. eutropha* H16. The amino acid sequences of the histidine kinase RegB were aligned using ClustalW (46). The results were visualized with Jalview (47). Identical residues are shaded according to the degree of conservation in all amino acid sequences. The RegB sequence alignment reveals conserved and functionally important motifs of RegB including the ubiquinone binding pocket (1), the site of phosphorylation with the active histidine residue, a threonine residue important for phosphatase activity (2) and the redox-active cysteine (3), which is essential for dimer and tetramer formation.

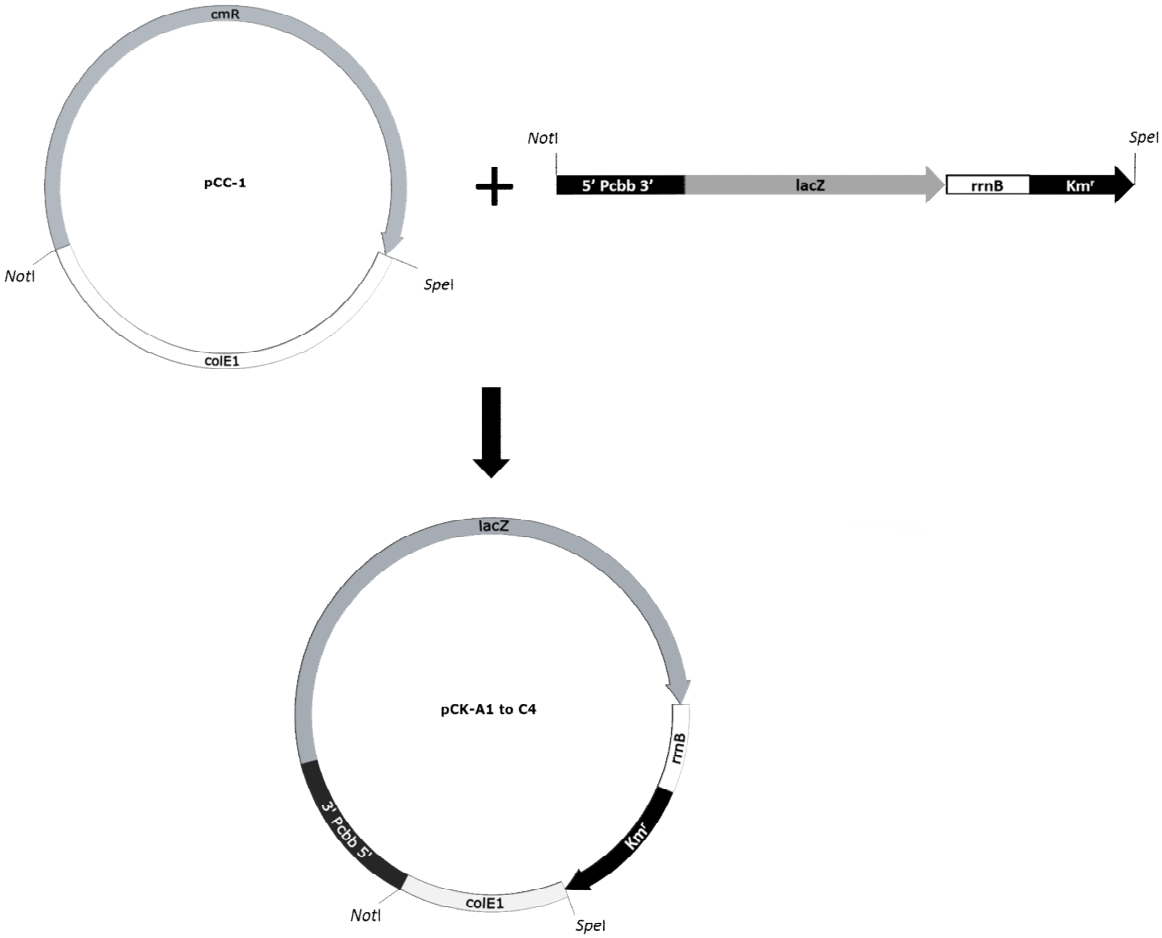

Figure S2: Illustration of basic vector design. pCC-1, encoding a colE1 *oriV* and Cm^r, and the PCR fragment encoding the particular *cbb* promoter, *lacZ*, *rrnB* and Km^r were combined via *Not*I/*Spe*I

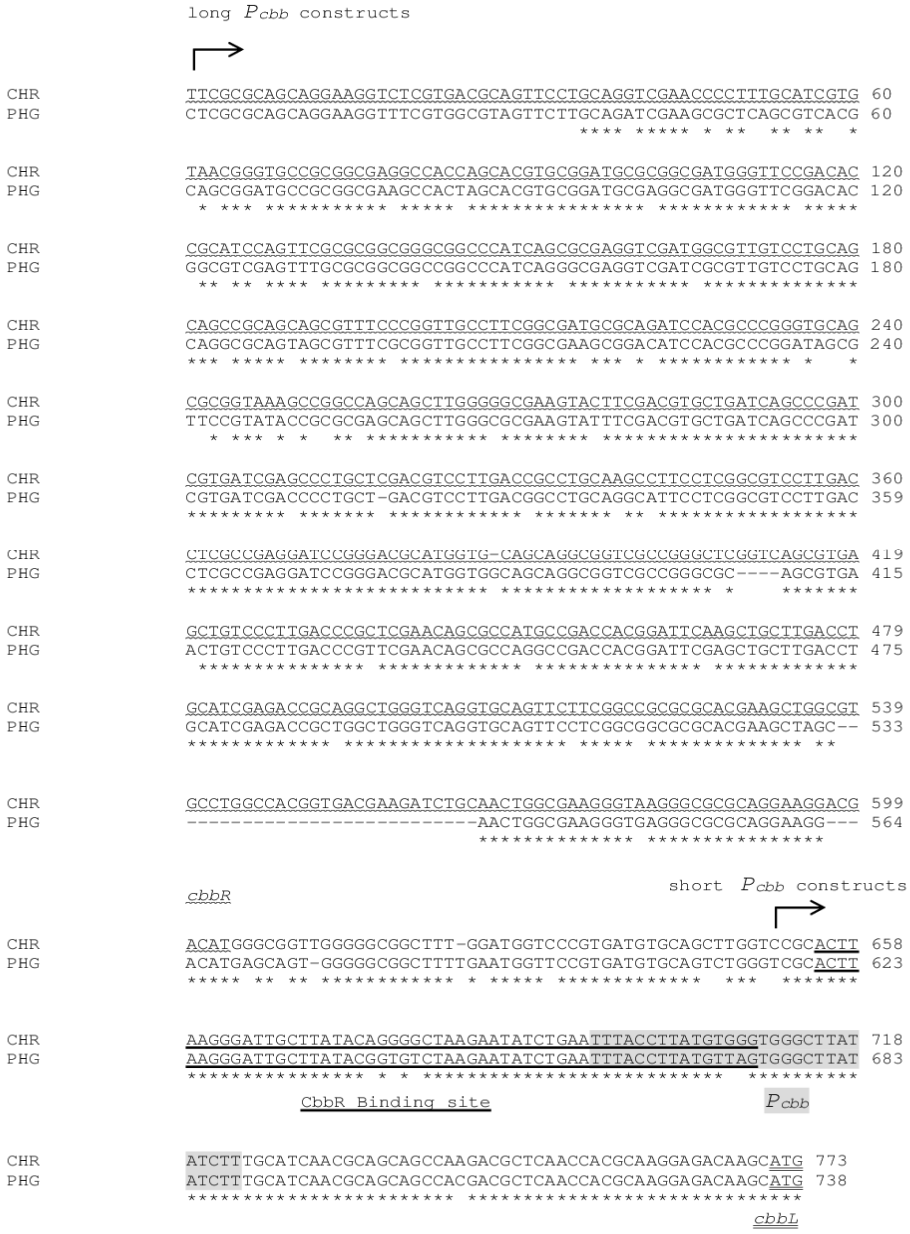

Figure S3: Nucleotide sequence alignment of the region covering 770 bp upstream of *cbbL*$_{C,P}$. Identical nucleotides are indicated by asterisks. The arrows indicate the primer binding sites for the short and long P_{cbb} constructs, P_{cbb} (shaded), CbbR binding site (black underline), *cbbL* start codon (double underline) and the coding sequence of *cbbR* (waved underline).

Table S1: Primers used in this study

Primer	Primer Sequence 5' to 3'
cbbR_rev_HindIII	AAGCTTTACCGCGACACCGGC
cbbRSD_fwd_XbaI	TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGTCGTCCTTCCTGCGC
RegA_fwd_NdeI	CATATGACCGACACCCTCACC
RegA_rev_HindIII	AAGCTTTACCGCGACACCGGC
PcbbL_fwd_KpnI	GGTACCTCGCACTTAAGGGATTGCTTATAC
UpPcbbL_rev_oe	GGTGGCGGAATCGAGGGCCATGCTTGTCTCCTTGCGTG
UpPcbbL_fwd-KpnI	GGTACCTTCGCGCAGCAGGAAGGT
UpPcbbL_Chr2_fwd	TGCTTGATGGTCTCGTTGCT
UpPccbL_PHG_fwd	TACTTGATCGTTTCATTGCTATCC
lacZ_OE_fwd	CACGCAAGGAGACAAGCATGACCATGATTACGGATTCA
LacZ_OE_rev	ATCAGGCTGAAAATCTTCTCTCATCCGCCAAAATTATTTTTGACACCAGACCAACTGGT
colE1_SpeI_fwd	ACTAGTCCCGTAGAAAAGATCAAAGGATCTTC
colE1_NotI_rev	GCGGCCGCATGTGAGCAAAAGGCCAGCAA
ShortPcbb_KpnI	GGTACCGAATTTACCTTATGT
LongPcbb_KpnI	GGTACCCTAAGAATATCTGAATT
UpcbbR rev LacZ oe	CAGTGAATCCGTAATCATGGTCATGGGCGGTTGGGGGCGGCTTTGGATGGTCC
UpcbbR fwd KpnI	GGTACCGTTCTCGTCATCCTTCATGAAGTCCA
Fwd cmR NotI_KpnI	GCGGCCGCGGTACCTCATGACGAATAAATACCTGTGAC
Rev cmR SpeI	ACTAGTTAACTGGCCTCAGGCATTT

4. Conclusions

4.1 Design of versatile plasmid vectors for the use in *R. eutropha* H16

Previous design of plasmid vectors intended for the use in *R. eutropha* H16 was based on minireplicons derived from broad-host-range plasmids pBBR1, RSF1010, RP4 or the megaplasmid pMOL28 derived from *R. metallidurans* CH43 (Lutte *et al.*, 2012; Sato *et al.*, 2013; Srinivasan *et al.*, 2003; Voss & Steinbüchel, 2006). All plasmid vectors designed on the basis of these minireplicons were able to replicate in *R. eutropha* H16, but exhibited significant plasmid loss during cultivation. In order to overcome this drawback metabolism-based and toxin/antidote plasmid addiction systems were applied on expression vectors to significantly reduce plasmid loss (Lutte *et al.*, 2012; Sato *et al.*, 2013; Srinivasan *et al.*, 2003; Voss & Steinbüchel, 2006).

Furthermore, several expression systems have been used to control expression of the gene of interest on plasmid vectors in *R. eutropha* H16. Most of these expression systems were based on native promoters derived from pyruvate, PHB, acetoin or *cbb* operons; a number of heterologous promoters such as P_{BAD}, P_{lac}, P_{lacUV5}, P_{tac} and P_{T7} was also applied (Barnard *et al.*, 2005; Bi *et al.*, 2013; Delamarre & Batt, 2006; Fukui *et al.*, 2011). Next to constitutive expression, several inducible expression systems were applied based on the particular regulatory elements including the IPTG-induced expression system based on the LacI repressor and an integrated lactose permease (LacY) function; the AraC repressor and the inducer L-arabinose; the TetR repressor responding to the inducer anhydrotetracycline (ATc); the XylS repressor responding to the inducer m-toluic acid or inducible expression systems based on the homologous *cbbL* and *phaP* promoters, which are induced under lithoautotrophic growth conditions or by phosphate depletion (Bi *et al.*, 2013; Li & Liao, 2015; Lutte *et al.*, 2012; Srinivasan *et al.*, 2002).

The construction of plasmid vectors described in chapters 1 and 3 aimed to increase the range of stably maintained expression vectors for the use in *R. eutropha* H16 based on one unified design (Figure 12 and 13). Therefore, a set of plasmid vectors was designed on the basis of minireplicons derived from broad-host-range plasmids pBBR1, RSF1010, RP4 and pSa covering a wide range of low or medium copy numbers. Based on significant plasmid loss, which was obtained for all plasmid vectors during fermentation of *R. eutropha* H16 transconjugants, the RP4 derived *par* region was included in vector design. In contrast to previous attempts aiming at plasmid stabilization, which were based on metabolism-based and toxin/antidote addiction systems, the RP4 *par* region encodes

a toxin/antitoxin system, a plasmid multimer resolution system and a plasmid segregation system. Accordingly, the RP4 *par* region was successfully applied to stabilize plasmid vectors based on RSF1010, RP4 and pSa minireplicons with plasmid retention rates of 100% over a time period of 96 hours and pBBR1 based plasmid vectors with a plasmid retention rate of at least 95% over a time period of 96 hours.

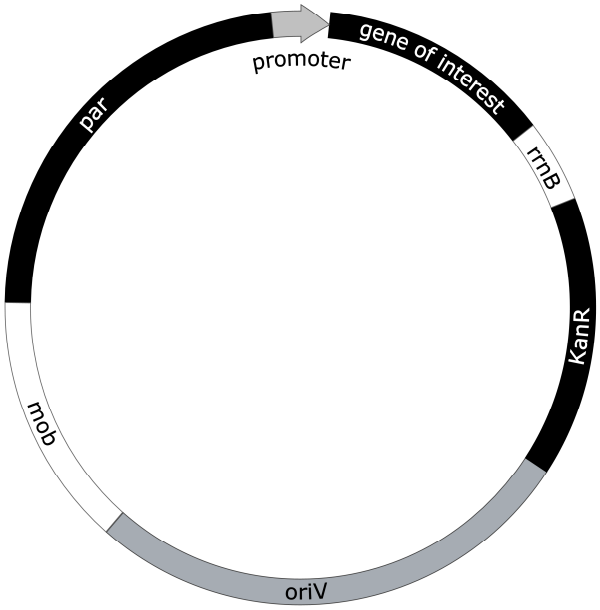

Figure 12: Basic plasmid design of expression vectors for the use in *R. eutropha* H16. All plasmid vectors encoded a promoter (P_{groEL}, P_{H16_B1772}, P_{h22b}, P_{f30}, P_{de33}, P_{n25}, P_{n26}, P_{g25}, P_{k28a}, P_{T5}, P_{k28b}, P_{h207} or P_{j5}), a gene of interest, the *rrnB* terminator sequence, a kanamycin resistance marker (Kan^r), an origin of replication (pBBR1, RSF1010, RP4, pSa^M or pSa), a RP4, RSF1010 or pBRR1 *mob* sequence and the RP4 *par* region.

The expression vector design also included the RP4 or RSF1010 derived *mob* sequences, which in comparison to the previously used pBBR1 derived *mob* sequence exhibited mobilization efficiencies that were increased by a factor of 50000 and 5000, respectively, promoting sufficient plasmid transfer during conjugation from *E. coli* S17-1 to *R. eutropha* H16. The substantially higher mobilization efficiency was also found to increase the probability of homologous recombination events significantly when engineering knock-in or knock-out strains of *R. eutropha* H16.

The expression systems applied in *R. eutropha* H16 on the basis of P_{groEL}, P_{H16_B1772}, P_{h22b}, P_{f30}, P_{de33}, P_{n25}, P_{n26}, P_{g25}, P_{k28a}, P_{T5}, P_{k28b}, P_{h207} or P_{j5} increased the range of feasible expression levels significantly. In this case, especially the bacteriophage T5 derived promoters were identified to be highly active and cover a wide range of promoter activities. Among these, the *j5* promoter was found to be the most active promoter characterized for the use in *R. eutropha* H16 so far. Next to the use of numerous promoters for constitutive expression, several inducible expression systems were previously used to control expression in *R. eutropha* H16 including AraC-based, TetR-based, XylS-based, lacI-based systems and the inducible expression systems based on *cbbL* and *phaP* promoters that were induced under lithoautotrophic growth conditions or by phosphate depletion (Bi *et al.*, 2013; Li & Liao, 2015; Lutte *et al.*, 2012; Srinivasan *et al.*, 2002). However, most of these inducible expression systems are not suitable for large scale fermentation processes or did not function in a satisfying manner. The AraC-based inducible system exhibits significant basal promoter activity, the TetR-based expression system cannot be used in large scale fermentations due to the antibiotic nature of the inducers and the lacI-based system used in *R. eutropha* H16 could not be fully induced (Bi *et al.*, 2013; Li & Liao, 2015). Moreover, the use of inducible expression systems based on *cbbL* and *phaP* promoters require an adaption of the fermentation process to provide inducing conditions (Lutte *et al.*, 2012; Srinivasan *et al.*, 2002). On the contrary, the inducible expression systems designed in chapter 3 on the basis of cumate or lacI regulatory elements and the *j5* promoter exhibited features suitable for the biotechnological application of *R. eutropha* H16 at a large scale such as tight regulation or highly tuneable and strong expression of the genes of interest. Moreover, full induction of the *j5* promoter could be achieved in case of the IPTG-induced expression system.

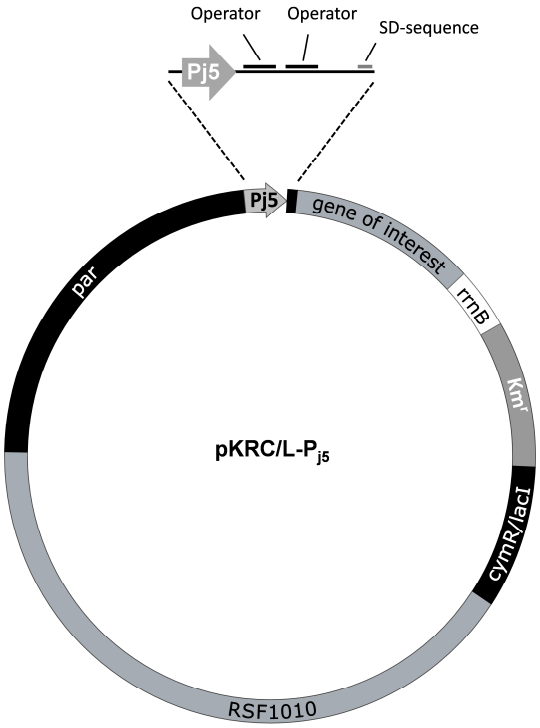

Figure 13: Design of plasmid-based inducible expression systems. Plasmid vectors encode the *j5* promoter followed by two operator sequences and the Shine-Dalgarno sequence, the gene of interest, an expression cassette encoding the *cymR* or *lacI* repressor genes according to application, the RP4 *par* region and the RSF1010 origin of replication.

Altogether, this newly constructed and versatile family of plasmid vectors exhibits many desired features promoting the biotechnological application of *R. eutropha* H16 as a production host. The applied minireplicons in combination with the RP4 or RSF1010 *mob* sequences promote high mobilization efficiencies and a wide range of plasmid copy numbers. In addition, all plasmid vectors could be stably maintained over a time period of at least 96 hours based on the RP4 *par* region. The expression range covered by the newly characterized bacteriophage T5 derived promoters and their combined application with IPTG or cumate regulatory elements enable tightly regulated and highly tuneable expression of the gene of interest in *R. eutropha* H16.

4.2 Transcription control of *cbb* operons in *R. eutropha* H16

The *cbb* operons encoded on chromosome two and pHG1 on the genome of *R. eutropha* H16 are almost identical. Both operons share a similar number of genes, only differing by genes encoding CbbR, the main *cbb* operon transcription regulator, and CbbB a formate dehydrogenase like protein. The transcription regulation of both *cbb* operons is executed by CbbR in response to the presence of the signal metabolite PEP, which controls transcription of the entire *cbb* operon from one σ^{70} promoter (P_{cbb}). The *cbb* promoter and CbbR binding sites are located in the same position on chromosome two and pHG1. CbbR binds in both cases upstream of P_{cbb} thereby inducing DNA bending and regulating transcription in response to cellular PEP levels. Transcription is induced in the presence of low PEP levels and repressed by high cellular PEP levels. As discussed in chapter 4 the pHG1 encoded *cbb* promoter was found to exhibit a significantly lower basal activity than P_{cbb} encoded on chromosome two, which is likely to result from differences in the nucleotide sequences located in the *cbb* core promoter region. In addition, the transcription regulation executed by CbbR, representing a feedback control with respect to the carbon-state of the cell, was found to be equally strong for pHG1 and chromosome encoded *cbb* promoters.

As described in chapter 4, the transcription of both *cbb* operons is also influenced by the global transcription regulation system RegA/RegB consisting of a membrane-bound histidine kinase (RegB) and the transcription regulator RegA. RegB is thought to phosphorylate RegA depending on the redox-state of the cell and ambient oxygen concentrations, which consequently forms dimers that activate transcription of target promoters. It could be demonstrated by analysing appropriate reporter constructs established in *E.coli* that the activity of both *cbb* promoters in *R. eutropha* H16 is significantly affected by RegA, inducing transcription from the *cbb* promoter independent of the signal metabolite PEP. Furthermore, RegA was found to exhibit a substantially higher influence on the pHG1 encoded *cbb* promoter compared to the chromosomal P_{cbb}, which is most likely the result of several minor deviations in the nucleotide sequences of both *cbb* control regions. Combined co-expression of CbbR and RegA affected *cbb* promoter activity in a PEP dependent manner and induced the highest *cbb* promoter activities reported so far. The transcription control executed by RegA and CbbR implements a cellular carbon-and energy-state feedback control for CO_2 fixation in *R. eutropha* H16.

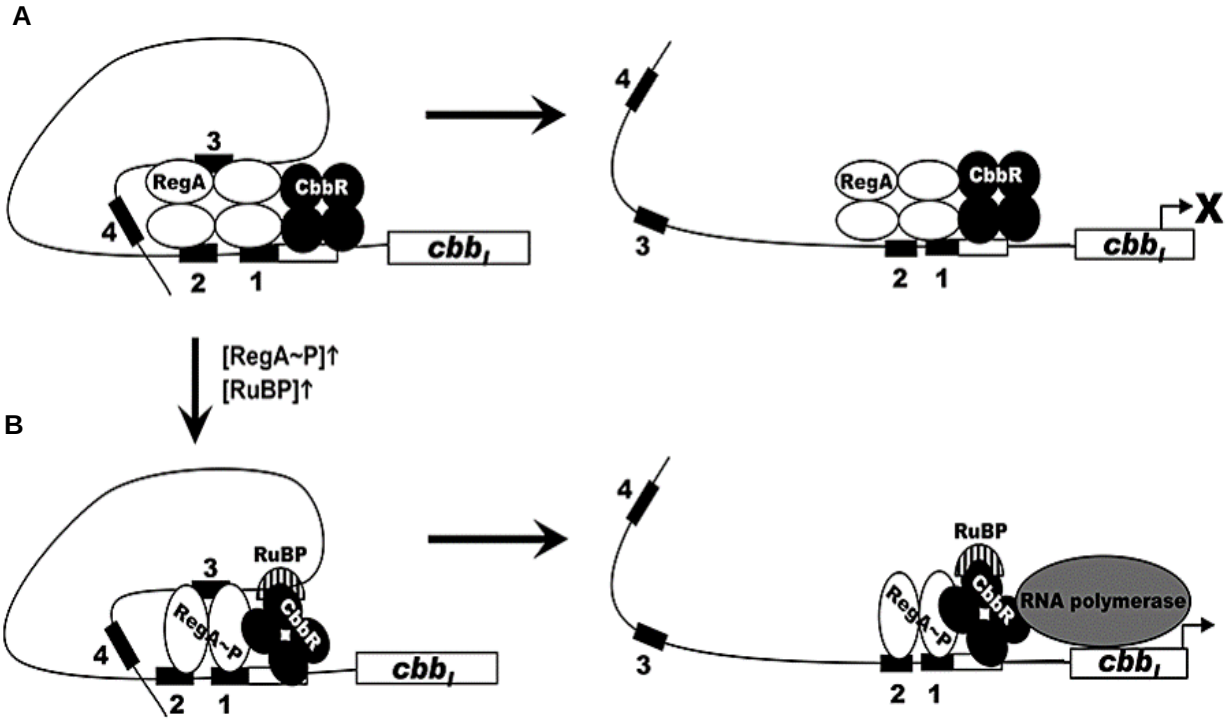

Figure 14: Transcription regulation of the *cbb* operon in *R. sphaeroides*. Elements include the RegA DNA binding sites (labelled 1 to 4); CbbR DNA binding site (white box); the signal metabolite RuBP; RNA polymerase and *cbbI* operon. **(A)** Unphosphorylated RegA and CbbR bind the particular DNA binding sites, RNA polymerase is not recruited. **(B)** Binding of RegA~P and CbbR, bound to RuBP, form a DNA loop to recruit RNA polymerase and induce *cbb* operon transcription. Image taken from Dangel & Tabita (2009).

In comparison, the transcription control of the *cbb* operon in *R. sphaeroides* involves CbbR-based regulation, which depends on ribulose-1,5-bisphosphate (RuBP) as signal metabolite, and RegA as part of the RegA/RegB system. The CbbR DNA binding sites are located directly upstream of P_{cbb}, which is controlled by CbbR dependent on RuBP levels. In addition, four RegA DNA binding sites are located upstream of *cbbI* enabling the binding of RegA and RegA~P. If sufficient amounts of phosphorylated RegA, RegA~P, and high levels of RuBP are present, transcription of the *cbb* operon is induced by a DNA loop formation recruiting RNA polymerase (Figure 14) (Dangel & Tabita, 2009). Moreover, protein-protein interactions between CbbR and RegA~P were found to support the formation of the transcription complex (Figure 15) (Dangel *et al*., 2014).

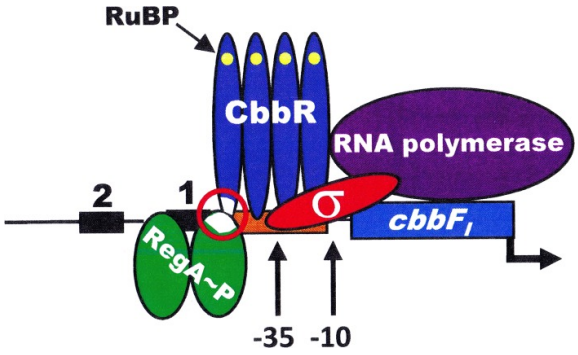

Figure 15: Transcription initiation complex at the *cbb* promoter in *R. sphaeroides*. Elements include the transcription regulator RegA~P (green), phosphorylated RegA; RegA binding sites 1 and 2; RuBP bound to the transcription regulator CbbR, which in turn is bound to the CbbR binding site (orange); the *cbb* promoter indicated by the -10 and -35 box; RNA polymerase (purple); the sigma 70 factor (red) and *cbbF*$_I$, the first gene of the *cbb* operon in *R. sphaeroides*. CbbR and RegA~P interactions are labelled in white and encircled in red. Image taken from Dangel *et al.* (2014).

Transcription of the *cbb* operon in *R. eutropha* H16 appears to occur similarly to the regulation described in *R. sphaeroides* including a carbon-and energy-state feedback control to manage the process of CO_2 fixation. However, further studies need to be performed to characterize the mechanism of *cbb* operon transcription regulation in *R. eutropha* H16 in more detail including the definition of features such as RegA DNA binding sites, RegA induced DNA looping or CbbR-RegA protein interactions.

5. Appendix

5.1 Additional expression vector building blocks for the use in *R. eutropha* H16

Plasmid replication elements

In the course of designing plasmid vectors for the use in *R. eutropha* H16, the minireplicon derived from the plasmid pSC101 was used to create a plasmid vector based on the unified plasmid design (Figure 12). However, the expression vector pKpSC101-P_{tac}-eGFP was not able to be maintained in *R. eutropha* H16 and did only replicate in *E. coli* cells. The pSC101 plasmid does follow a narrow host range replication that is most probably constrained to *E. coli* and closely related bacteria (Miller *et al.*, 1995). Replication of pSC101 starts unidirectional from an unique origin based on the plasmid encoded RepA protein, iteron sequences, AT-rich regions and the host protein DnaA (Kües & Stahl, 1989).

Stabilization and maintenance of plasmid vectors

Next to the RP4 derived *par* region, encoding the DNA gyrase inhibiting toxin parE and the antidote parD, the R100 derived PemK/PemI toxin/antidote system was used to promote plasmid stability in *R. eutropha* H16. The Pem system delays cell division by binding of PemK to DnaB type proteins (Ruiz-Echevarría *et al.*, 1995). The activity of the toxin, PemK, is neutralized in the presence of the antidote PemI, which forms a complex with PemK (Jensen & Gerdes, 1995).

The Pem system was included in vectors pCM_PT7_RSF1010_eGFP_PemIk_lacI and pCM_PT7_RSF1010_eGFP_PemIk_ΔlacI, which were successfully maintained in *R. eutropha* H16 (Hagen, 2015). However, long term plasmid stability assays on the basis of the PemK/PemI toxin/antidote system were not yet performed. Since the RP4 derived toxin/antidote system acts on different targets than the Pem system, both toxin/antidote systems could in theory be used on different plasmids to maintain two plasmids in one cell with respect to plasmid incompatibility.

Expression systems

The design of inducible expression systems based on the *j5* promoter, *egfp* as reporter gene and a RSF1010 vector backbone included two sets of inducible expression systems that obtained different setups of promoter and operator sequences including an operator-promoter-operator and a promoter-operator-operator setup (Figure 16). The following regulatory elements were used for the construction of the inducible expression systems: the lac system derived from *E. coli* including a lactose permease function (lacY) and the inducer IPTG, the cumate system including the cumate repressor (cymR) derived from *P. pudita*, hydroxycinnamate (hca) system including hcaR (*Reut_B4874*) from *Ralstonia eutropha* JMP134 , the PobR (*H16_B2287*) repressor from the p-coumaric acid degrading operon in *R. eutropha* H16 and the MobR (*BAF34929.1*) repressor of the 3-hydroxybenzoate degrading operon in *Comamonas testosteroni* (Bertani *et al.*, 2001; Choi *et al.*, 2010; Hiromoto *et al.*, 2006; Parke & Ornston, 2003).

The cumate- and IPTG-induced expression systems based on the operator-promoter-operator setup exhibited satisfying inducible features, but showed a high rate of basal promoter activity under uninduced conditions. As a consequence the promoter-operator-operator setup was applied for plasmid vector construction, which exhibited promising features for the cumate-and IPTG-induced expression systems and are described in more detail in chapter 3.

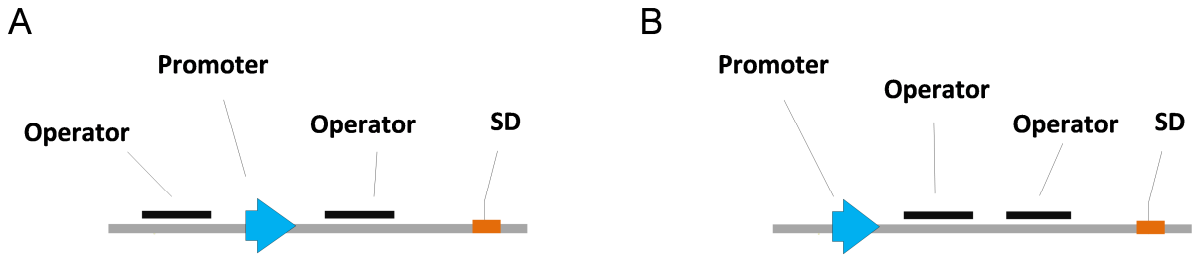

Figure 16: Design of inducible expression cassettes. The Shine-Dalgarno sequence (SD) is indicated in red, the promoter in blue, operator sequence are indicated in black (A) Setup based on an operator, promoter and operator sequence. (B) Setup based on a promoter, operator and operator sequence.

The hydroxycinnamate (hca) system derived from *R. eutropha* JMP134 was based on the repressor HcaR (*Reut_B4874*) (Parke & Ornston, 2003). This MarR-type transcription regulator directly controls the expression of the *hca* operon in *R. eutropha* JMP134. However, the signal molecule inducing *hca* operon transcription by binding to hcaR is not known (Parke & Ornston, 2003).

Expression vectors designed on the basis of the *hca* inducible expression system in an operator-promoter-operator setup (Table 1) were strongly repressed in *R. eutropha* H16, but could not be induced using p-coumarate as inducer. It is possible that other hydroxycinnamates like ferulate and caffeate or thioester intermediates act as inducers of the *hca* operon (Parke & Ornston, 2003).

The PobR (*H16_B2287*) repressor from the p-coumaric acid degrading operon in *R. eutropha* H16 and the MobR (*BAF34929.1*) repressor of the 3-hydroxybenzoate degrading operon in *C. testosteroni* were used in an operator-promoter-operator setup and in a promoter-operator-operator setup (Bertani *et al.*, 2001; Hiromoto *et al.*, 2006). However, *egfp* expression could not be controlled in *R. eutropha* H16 on the basis of the PobR or MobR transcription regulators and several putative operator sites (Table 1). The effect of the inducers p-coumaric acid and 3-hydroxybenzoate could not be assessed due to the strong basal eGFP expression. Accordingly, inducible expression systems based on MobR, PobR or HcaR were not used for further studies.

Table 1: Transcription regulators and the particular operator sequences used to construct plasmid-based inducible expression systems.

Transcription regulator	Operator sequence	Functional
HcaR	CTACTTGATATGTCAGGAAGCCTGATACTATA	Yes
MobR	TACTATTTGTGTGCGGACTGA	No
PobR	TTGGCGGGTCTCCGCCGACT	No
PobR	TTTACCATCGATGTTCCGATTGTCCT	No
PobR	TCTTTAGCGGCAGAAGACCGATAACC	No

5.2 Design of expression systems promoting protein secretion in *R. eutropha* H16

The approach for establishing protein secretion in *R. eutropha* H16 including signal sequence identification and selection, plasmid design and screening assays were elaborated by Steffen Gruber. However, comprehensive wet-lab work that established the basis for protein secretion in *R. eutropha* H16 was performed by Eva Thaler in terms of her Master Thesis (Thaler, 2015). The ability to secret the protein of interest to the environment is of significant biotechnological interest since protein secretion improves cost factors based on the purification processes, simplifies the harvesting process, decreases contamination with cellular compounds and decreases the chances of proteolytic degradation (Yoon *et al.*, 2010; Zhang *et al.*, 2006). Consequently, a set of plasmids was designed to establish protein secretion properties on the basis of sec and tat secretory pathways in recombinant strains of *R. eutropha* H16 (Figure 17 and 18).

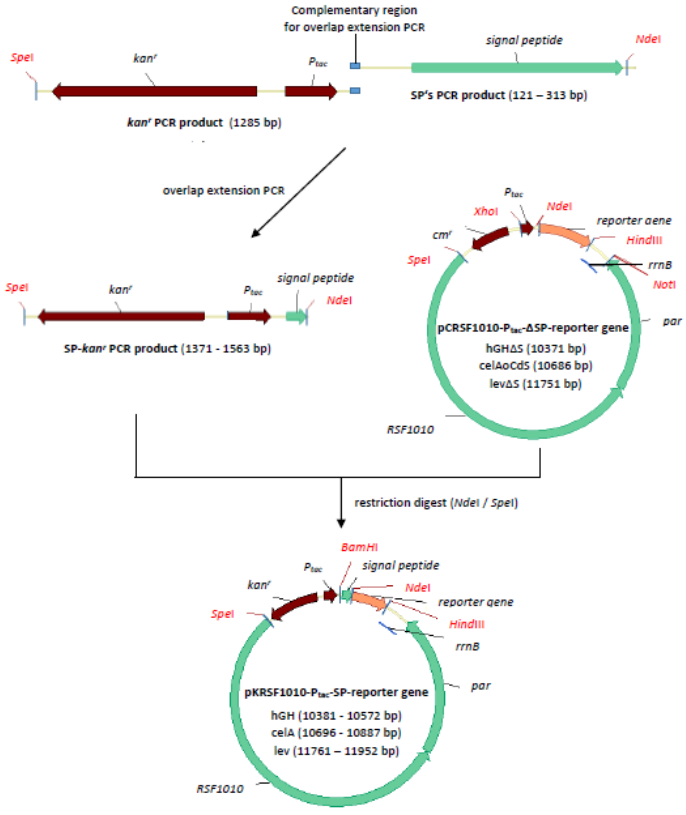

Figure 17: Construction of the secretion plasmids pKRSF1010-P_{tac}-SP-hGH, pKRSF1010-P_{tac}-SP-celA and pKRSF1010-P_{tac}-SP-lev. For details see the Master thesis of Eva Thaler (Thaler, 2015).

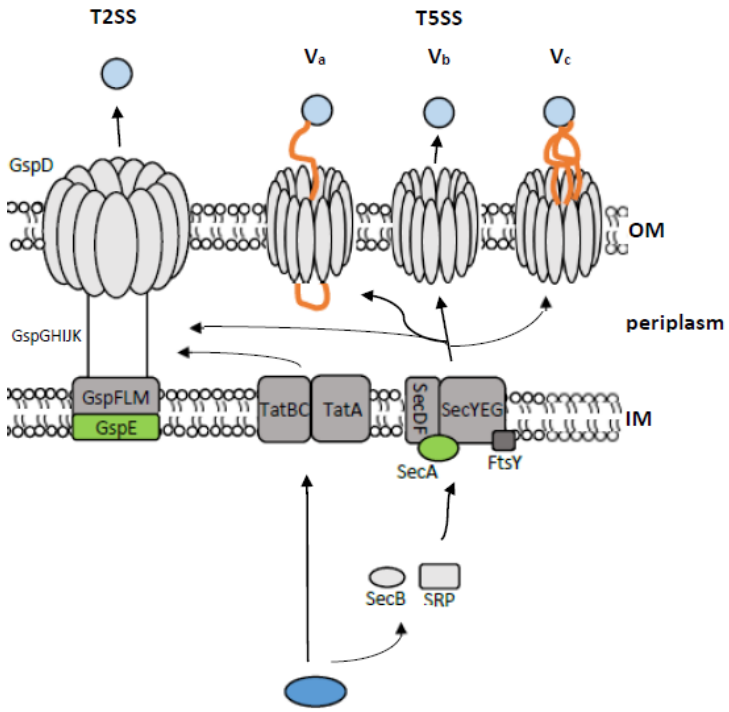

Figure 18: Tat- and Sec-dependent protein secretion. Protein secretion appears in two steps, the precursor proteins (blue circle) containing the signal sequence are exported across the inner membrane (IM). The T2SS and T5SS substrates are targeted via N-terminal signal sequences that enable the translocation by the Sec- or Tat-dependent pathway. The signal sequences are cleaved in the periplasm to cross the outer membrane (OM) through the T2SS or T5SS apparatus. ATPases are labelled in green and the translocation apparatus in grey. Image taken from Thaler (2015).

The identification of potential signal sequences promoting protein secretion were obtained from the plant pathogen *Ralstonia solanacearum* FQY-4 and *R. eutropha* H16 based on previously performed studies, which investigated the secretory capacities of *R. solanacearum* FQY-4 (Zuleta, 2001). On the basis of this work, 11 tat and 10 sec signal sequences were selected for plasmid vector design (Table 2).

Table 2: Signal peptides (SP) used in the study conducted by Thaler (2015).

No.	Name	Size	Locus[1]
			SP's for sec-dependent pathway
S1	pehB	79 aa	F504_1633
S2	Pme	26 aa	F504_3589
S3	Egl	30 aa	F504_3606
S4	cbhA	46 aa	F504_4041
S5	Tek	34 aa	F504_4201
S6	Aac	28 aa	F504_2493
S7	treA	45 aa	F504_3718
S8	pqaA	23 aa	F504_3605
S9	F504_4738	27 aa	F504_4738
S10	F504_2783	20 aa	F504_2783
			SP's for Tat-pathway
T1	NosL	31 aa	F504_4829
T2	F504_2199	38 aa	F504_2199
T3	F504_2437	35 aa	F504_2437
T4	RlpB	30 aa	F504_2669
T5	F504_2793	27 aa	F504_2793
T6	amiC	48 aa	F504_2485
T7	nasF	41 aa	F504_402
T8	iorB2	42 aa	F504_1888
T9	ReH16NosZ	45 aa	PHG252
T10	pehC	57 aa	F504_4386
T12	RscNosZ	51 aa	F504_4824

1 gene locus of exoproteins in *R. solanacearum* FQY-4, except for T9: gene locus in *R. eutropha* H16

The expression plasmids were designed based on a RSF1010 backbone encoding the constitutive *tac* promoter and the reporter genes cellulase A (*celA*), levanase (*lev*) and the human growth hormone (*hGH*) (Figure 17).

Even though, protein secretion of HGH and Lev was only detectable in small amounts or was not feasible, secretion of CelA was accomplished on the basis of several signal sequences (Thaler, 2015). This is partially due to the stress conditions that were already observed to occur during plasmid vector assembly in *E. coli* strains, which caused significant mutation or deletion events (Thaler, 2015). Nevertheless, the majority of plasmid vectors could be assembled and were established in *R. eutropha* H16. The detection of Lev and HGH in the supernatant proved to be less efficient than CelA detection. On the one hand, Lev and HGH appear to be less efficiently secreted by *R. eutropha* H16 than CelA, on the other hand the Congo red assay used to detect secreted CelA is substantially more sensitive than methods used to detect Lev and HGH (Thaler, 2015). Nonetheless, a basis for protein secretion in *R. eutropha* H16 could be established that is likely to be further improved based on inducible expression of reporter genes and engineering aimed at the secretion apparatus of *R. eutropha* H16.

5.3 High-cell-density fermentation processes with *R. eutropha* H16

The use of *R. eutropha* H16 as production host in high-cell-density fermentation processes under lithoautotrophic and heterotrophic conditions has been established on the basis of several protocols yielding cell densities up to 230 g/l (Ryu *et al.*, 1997; Srinivasan *et al.*, 2002). Advantages for using *R. eutropha* H16 in high-cell-density fermentation processes include improved operating costs, increased productivity and higher product concentrations (Andersson *et al.*, 1994; Chen *et al.*, 1992). Accordingly, fermentation protocols were elaborated to establish fed-batch fermentations on the basis of *R. eutropha* H16 transconjugants carrying the plasmid pKRC-P_{j5}-estA.

Single colonies of *R. eutropha* H16 (pKRC-P_{j5}-estA) were used to inoculate liquid fermentation media and the ONCs were grown to suitable cell densities to inoculate Biostat B fermenters at an OD_{600} of 0.5. The Biostat B fermenters were used at 28°C and a cascade based on 1.5 lpm air supply including stirring at 300 rpm, which was automatically increased to 1000 rpm based on the oxygen saturation. The oxygen saturation was set at 98 %. The feed solution was added at a rate of 12.5 ml/h when cell growth began to slow down, typically after approximately 16 - 24 h after inoculation. In total the fermentation process was repeated four times in duplicate. However, for unknown reasons the cell density of the fermented culture reached only an OD_{600} of 14 and stopped growing.

Composition of fermentation medium:

Component	Culture medium	Feed solution
Fructose	20 g/l	700 g/l
$(NH_4)_2SO_4$	4 g/l	
$MgSO_4 \cdot 7H_2O$	0.2 g/l	
KH_2PO_4	5.5 g/l	
NaCl	2.5 g/l	
Citric acid	1.7 g/l	
Trace element solution	10 ml/l	

Composition of the trace element solution:

$FeSO_4 \cdot 7H_2O$	10 g/l
$ZnSO_4 \cdot 7H_2O$	2.25 g/l
$CuSO_4 \cdot 5H_2O$	1 g/l
$MnSO_4 \cdot 5H_2O$	0.5 g/l
$CaCl_2 \cdot 2H_2O$	2 g/l
H_3BO_3	62 mg/l
$(NH_4)_2MnoO_4$	108 mg/l

Dissolved in 35% HCl

5.4 Alternative approaches for the characterization of *cbb* operon transcription

Alternative reporter enzyme

Primarily, an alternative reporter gene was selected for the quantification of *cbb* promoter activity as performed in chapter 4, which allowed for the detection of promoter activity in low oxygen or anaerobic environments. The small sized flavin mononucleotide based fluorescent proteins (FbFP) are capable of oxygen-independent maturation of fluorescence, which promotes their use as a reporter for promoter activity also in low oxygen or anaerobic environments (Mukherjee *et al.*, 2013). The application of FbFPs was not considered for further use since comparative analysis of *cbb* promoter activity on the basis of β-galactosidase as a reporter enzyme proved to be suitable for the conducted study (Chapter 4).

Nucleotide sequence PpfbFP:

atgatcaacgcaaaactcctgcaactgatggtcgaacattccaacgatggcatcgttgtcgccgagcaggaaggcaatgagagcatccttatctacgtcaacccggccttcgagcgcctgaccggctactgcgccgacgatattctctatcaggacgcacgttttcttcagggcgaggatcacgaccagccgggcatcgcaattatccgcgaggcgatccgcgaaggccgcccctgctgccaggtgctgcgcaactaccgcaaagacggcagcctgttctggaacgagttgtccatcacaccggtgcacaacgaggcggaccagctgacctactacatcggcatccagcgcgatgtcacagcgcaagtattcgccgaggaaagggttcgcgagctggaggctgaagtggcggaactgcgccggcagcagggccaggccaagcactga

Amino acid sequence PpfbFP:

M I N A K L L Q L M V E H S N D G I V V A E Q E G N E S I L I Y V N P A F E R L T G Y C A D D I L Y Q D A R F L Q G E D H D Q P G I A I I R E A I R E G R P C C Q V L R N Y R K D G S L F W N E L S I T P V H N E A D Q L T Y Y I G I Q R D V T A Q V F A E E R V R E L E A E V A E L R R Q Q G Q A K H Stop

Co-expression of *cbbR* and *regA*

Transcription regulation of co-expressed *cbbR* and *regA* used for the detection of *cbb* promoter activity as described in chapter 4 was originally attempted on the basis of the *H16_B1772* promoter. However, the co-expression of *cbbR* and *regA* was found to be significantly too strong, which negatively influenced the reproducibility of β-galactosidase activities due to significant stress exerted on the cells. These stress conditions did lead to mutations in the plasmid vectors prohibiting reliable results.

A set of plasmid vectors encoding the upstream region of the RegA/RegB operon found in *R. eutropha* H16 was also intended for characterization due to the autoregulatory nature of the systems identified in *R. sphaeroides* and *R. capsulatus*. This was not yet examined in *R. eutropha* H16.

6. Supplementary Information

List of strains forwarded to the IMBT culture collection

Strain	Description	IMBT
E. coli TOP10 (pKRSF1010-P_{tac}-Ru1)	Km^r, P_{tac}, *estA*, *par*, *mob*, RSF1010 origin of replication	7695
E. coli TOP10 (pKSa-P_{tac}-egfp-mob-pBBR1)	Km^r, P_{tac}, *egfp*, *par*, mobilization sequence *mob* from the pBBR1MCS-5 plasmid, pSa origin of replication	7696
E. coli TOP10 (pKSa-P_{tac}-egfp-mob-RSF1010)	Km^r, P_{tac}, *egfp*, *par*, mobilization sequence *mob* from the RSF1010 plasmid, pSa origin of replication	7697
E. coli TOP10 (pKSa-P_{tac}-egfp)	Km^r, P_{tac}, *egfp*, partition region *par* from the RP4 plasmid, mobilization sequence *mob* from the RP4 plasmid, pSa origin of replication	7698
E. coli TOP10 (pKRep-P_{tac}-egfp)	Km^r, P_{tac}, *egfp*, *par*, RP4 *mob*, pBBR1 origin of replication	7699
E. coli TOP10 (pKRP4-P_{tac}-egfp)	Km^r, P_{tac}, *egfp*, *par*, RP4 *mob*, RP4 origin of replication	7700
E. coli TOP10 (pKSaM-P_{tac}-egfp)	Km^r, P_{tac}, *egfp*, *par*, RP4 *mob*, pSa origin of replication, contains a mutation in the RepA protein	7701
E. coli TOP10 (pKRSF1010-P_{tac}-egfp)	Km^r, P_{tac}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7702
E. coli TOP10 (pKRSF1010-P_{lac}-egfp)	Km^r, P_{lac}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7703
E. coli TOP10 (pKRSF1010-P_{T5}-egfp)	Km^r, P_{T5}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7704

E. coli TOP10 (pKRSF1010-P_{j5}-egfp)	Km^r, P_{j5}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7705
E. coli TOP10 (pKRSF1010-P_{k28a}-egfp)	Km^r, P_{k28a}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7706
E. coli TOP10 (pKRSF1010-P_{k28b}-egfp)	Km^r, P_{k28b}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7707
E. coli TOP10 (pKRSF1010-P_{n25}-egfp)	Km^r, P_{n25}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7708
E. coli TOP10 (pKRSF1010-P_{n26}-egfp)	Km^r, P_{n26}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7709
E. coli TOP10 (pKRSF1010-P_{h22b}-egfp)	Km^r, P_{h22b}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7710
E. coli TOP10 (pKRSF1010-P_{de33}-egfp)	Km^r, P_{de33}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7711
E. coli TOP10 (pKRSF1010-P_{g25}-egfp)	Km^r, P_{g25}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7712
E. coli TOP10 (pKRSF1010-P_{h207}-egfp)	Km^r, P_{h207}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7713
E. coli TOP10 (pKRSF1010-P_{f30}-egfp)	Km^r, P_{f30}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7714
E. coli TOP10 (pKRSF1010-P_{H16_B1772}-egfp)	Km^r, P_{H16_B1772}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7715
E. coli TOP10 (pKRSF1010-P_{groEL}-egfp)	Km^r, P_{groEL}, *egfp*, *par*, RSF1010 *mob* and origin of replication	7716
E. coli TOP10 (pKRSF1010Δegfp)	Km^r, P_{tac}, *par*, RSF1010 *mob* and origin of replication, deleted *egfp*	7717
E. coli TOP10 (pK470MobRP4)	Km^r, P_{tac}, *mob*, *colE1*	7718

E. coli TOP10 (pInt_lacY_phaC)	pK470MobRP4, *lacY* gene, P_{H16_B1772}, two phaC homologous regions	7719
E. coli TOP10 (pInt_lacY_phaC_loxP)	pK470MobRP4, *lacY* gene, P_{H16_B1772}, two phaC homologous regions, loxP sites	7720
E. coli TOP10 (pCM_Cre)	Cm^r, P_{tac}, *mob, colE1, cre, cymR*	7721
E. coli TOP10 (pKRL-P_{j5}-egfp)	Km^r, P_{j5}, *egfp*, *par*, *lacI*, RSF1010 *mob* and origin of replication	7722
E. coli TOP10 (pKRC-P_{j5}-egfp)	Km^r, P_{j5}, *egfp*, *par*, *cymR*, RSF1010 *mob* and origin of replication	7723
E. coli TOP10 (pKRC-P_{j5}-estA)	Km^r, P_{j5}, *estA*, *par*, *cymR*, RSF1010 *mob* and origin of replication	7724
E. coli TOP10 (pCC-1)	Cm^r, colE1 origin of replication	7725
E. coli TOP10 (pCK-A1)	Km^r, short chromosomal P_{cbb} , *lacZ,* colE1 origin of replication	7726
E. coli TOP10 (pCK-A2)	Km^r, long chromosomal P_{cbb} , *lacZ*, colE1 origin of replication	7727
E. coli TOP10 (pCK-A3)	Km^r, short chromosomal P_{cbb} , *lacZ*, *cbbR*, colE1 origin of replication	7728
E. coli TOP10 (pCK-A4)	Km^r, long chromosomal P_{cbb} , *lacZ*, *cbbR*, colE1 origin of replication	7729
E. coli TOP10 (pCK-A5)	Km^r, short chromosomal P_{cbb} , *lacZ, regA*, colE1 origin of replication	7730
E. coli TOP10 (pCK-A6)	Km^r, long chromosomal P_{cbb} , *lacZ, regA*, colE1 origin of replication	7731
E. coli TOP10 (pCK-A7)	Km^r, short chromosomal P_{cbb} , *lacZ, cbbR, regA,* colE1 origin of replication	7732
E. coli TOP10 (pCK-A8)	Km^r, long chromosomal P_{cbb} , *lacZ, cbbR, regA*, colE1 origin of replication	7733

E. coli TOP10 (pCK-B1)	Km^r, short pHG1 P_{cbb} , *lacZ*, colE1 origin of replication	7734
E. coli TOP10 (pCK-B2)	Km^r, long pHG1 P_{cbb} , *lacZ,* colE1 origin of replication	7735
E. coli TOP10 (pCK-B3)	Km^r, short pHG1 P_{cbb} , *lacZ, cbbR*, colE1 origin of replication	7736
E. coli TOP10 (pCK-B4)	Km^r, long pHG1 P_{cbb} , *lacZ, cbbR*, colE1 origin of replication	7737
E. coli TOP10 (pCK-B5)	Km^r, short pHG1 P_{cbb} , *lacZ, regA*, colE1 origin of replication	7738
E. coli TOP10 (pCK-B6)	Km^r, long pHG1 P_{cbb} , *lacZ, regA*, colE1 origin of replication	7739
E. coli TOP10 (pCK-B7)	Km^r, short pHG1 P_{cbb} , *lacZ, cbbR, regA*, colE1 origin of replication	7740
E. coli TOP10 (pCK-B8)	Km^r, long pHG1 P_{cbb} , *lacZ, cbbR, regA*, colE1 origin of replication	7741
E. coli TOP10 (pCK-C1)	Km^r, P_{cbbR} , *lacZ*, colE1 origin of replication	7742
E. coli TOP10 (pCK-C2)	Km^r, P_{cbbR} , *lacZ, cbbR*, colE1 origin of replication	7743
E. coli TOP10 (pCK-C3)	Km^r, P_{cbbR} , *lacZ, regA,* colE1 origin of replication	7744
E. coli TOP10 (pCK-C4)	Km^r, P_{cbbR} , *lacZ*, *cbbR*, *regA*, colE1 origin of replication	7745
E. coli TOP10 (pKR-P_{j5}-egfp-mobR)	Km^r, P_{j5}, *egfp*, *par*, *mobR*, RSF1010 *mob* and origin of replication	7746
E. coli TOP10 (pKR-P_{j5}-egfp-pobR)	Km^r, P_{j5}, *egfp*, *par*, *pobR*, RSF1010 *mob* and origin of replication	7747
E. coli TOP10 (pKR-P_{j5}-egfp-hcaR)	Km^r, P_{j5}, *egfp*, *par*, *hcaR*, RSF1010 *mob* and origin of replication	7748
R. eutropha RS1	H16 ΔphaCΩPH16_B1772lacY	7749

List of primers used in this study

#[a]	Primer	Sequence 5' to 3'
692	Fwd cmR NotI_KpnI	gcggccgcggtacctcatgacgaataaatacctgtgac
693	KanR-SpeI-rev	cggactagtgtctgacgctcagtggaacgaa
694	Pj5-lacO-fwd-1	ggataacaattcgattcggaattgtgagcggataacaattcaattcgagctcggtacccg
695	Pj5-lacO-fwd-2	attgacacaggtggaaatttagaatatactgggaattgtgagcggataacaattcgattc
696	Pj5-lacO-NotI-fwd-3	gcggccgcaaaaaccgttattgacacaggtggaaa
697	PT5j5lacONotIfwd	cggccgcaaccgttattgacatgtgagcggataacaatttatactgaattcgagctc
698	Pj5-mobOO-fwd-2	attgacacaggtggaaatttagaatatactgtactatttgtgtgcggactgagattc
699	Pj5-mobO-3-NotI-fwd-3	gcggccgcctactatttgtgtgcggactgaaaaaaccgttattgacaca
700	Pj5-mobO-3-fwd-2	aaaaaccgttattgacacaggtggaaatttagaatatactgtactatttgtgtgcggact
701	Pj5-mobO-3-fwd-1	tactatttgtgtgcggactgaaattcgagctcggtacccg
702	pobR-oe-fwd	agattttcaggagctaaggaagctaaaatgattaactctgcactgccaaac
703	pobR-oe-TT7-rev	cccttggggcctctaaacgggtcttgaggggttttttgtcagcctgcgggcgtctgctcc
704	Pj5-xyOO-NotI-fwd-3	gcggccgcaaaaaccgttattgacacaggtggaaatttagaatatactgaaca
705	Pj5-cyOO-fwd-2	tttagaatatactgaacaaacagacaatctggtctgtttatcttatagattcaacaaaca
706	Pj5-cyOO/T7pol-fwd-1	atagattcaacaaacagacaatctggtctgtttgtattataaattcgagctccgtacccg
707	TT7-1-rev	ccgtttagaggccccaaggggttatgctagtgcatgcagctctcatccgccaaaacagcc
708	TT7-StuI-2-rev	aggcctcaaaaaacccctcaagacccgtttagaggccccaagggg
709	RegaoerrnB_Fw	gggaaagcggccggtgtcgcggtaaagcttggctgttttggcggatgaga
710	UpcbbR fwd KpnI	ggtaccgttctcgtcatccttcatgaagtcca
711	UpcbbR rev LacZ oe	cagtgaatccgtaatcatggtcatgggcggttgggggcggctttggatggtcc
712	UpRegab_rev_LacZ_oe	cagtgaatccgtaatcatggtcatggcgcgagtgtatcaatgcggccg
713	Cbbr oe DELATG fwd	gcaggaaggacgacaagggcggtt
714	Cbbr oe DELATG rev	aaccgcccttgtcgtccttcctgc

715	LongPcbb_KpnI	ggtaccctaagaatatctgaatt
716	ShortPcbb_KpnI	ggtaccgaatttaccttatgt
717	Pj5-mobOO-fwd-1	tgtgcggactgagattctactatttgtgtgcggactgaaattcgagctcggtacccg
718	Pj5-pobOO-fwd-2	attgacacaggtggaaatttagaatatactgttggcgggtctccgccgactgattc
719	Pj5-pobOO-fwd-1	tctccgccgactgattcttggcgggtctccgccgactaattcgagctcggtacccg
720	5´Int phaC1 fwd	atagcatctccccatgcaaagtgc
721	3´Int phaC1 rev	cggatacgatgacaacgtcagtca
722	pobR-oe-fwd	agattttcaggagctaaggaagctaaaatgaaacctgtcccgacgtactctc
723	pobR-oe-TT7-rev	cccttggggcctctaaacgggtcttgaggggttttttgtcagcccgccgcatccgcgggc
724	Pj5-pobO-3-NotI-fwd-3	gcggccgccttggcgggtctccgccgactaaaaaccgttattgacaca
725	Pj5-pobO-3-fwd-2	aaaaaccgttattgccacaggtggaaatttagaatatactgcttggcgggtctccgccga
726	Pj5-pobO-3-fwd-1	cttggcgggtctccgccgactaattcgagctcggtacccg
727	colE1_SpeI_fwd	actagtcccgtagaaaagatcaaaggatcttc
728	colE1_NotI_rev	gcggccgcatgtgagcaaaaggccagcaa
729	lacZ_OE_fwd	cacgcaaggagacaagcatgaccatgattacggattca
730	LacZ_OE_rev	atcaggctgaaaatcttctctcatccgccaaaattatttttgacaccagaccaactggt
731	UpcbbL_oe_BsFbFP_rev	atgattgaaaactagccatgcttgtctccttgcgtggttg
732	TT:oe_BsFbFP_fwd	gaaaagcttctcgagtgaggctgttttggcggatgag
733	UpRegAB_oe_BsFbFP_rev	ccaaatgattgaaaactagccatggcgcgagtgtatcaatgcgg
734	Upcbbl_oe_PpFbFP_Rev	ggagttttgcgttgatcatgcttgtctccttgcgtggttg
735	TT_oe_PpFbFP_fwd	ggccaggccaagcactgaggctgttttggcggatgcgaga
736	UpRegAB_oe_PpFbFP_rev	caggagttttgcgttgatcatggcgcgagtgtatcaatgcggccg
737	Pj5-pobO-1-NotI-fwd-3	gcggccgctttaccatcgatgttccgattgtcctaaaaaccgttattgacaca
738	Pj5-pobO-1-fwd-2	aaaaaccgttattgacacaggtggaaatttagaatatactgtttaccatcgatgttccga
739	Pj5-pobO-1-fwd-1	tttaccatcgatgttccgattgtcctaattcgagctcggtacccg
740	Pj5-pobO-2-NotI-fwd-3	gcggccgctctttagcggcagaagaccgataaccaaaaaccgttattgacaca
741	Pj5-pobO-2-fwd-1	tctttagcggcagaagaccgataaccaattcgagctcggtacccg

742	Primer PCIVB1M_revoeRegA	ggggtgagggtgtcggtcatgattggcttcctcgagagacct
743	Pj5-CymO-NotI-fwd-3	gcggccgcaacaaacagacaatctggtctgtttgtattataaaaaaccgttattgacaca
744	Pj5-CymO-fwd-2	aaaaaccgttattgacacaggtggaaatttagaatatactgaacaaacagacaatctggt
745	Pj5-CymO-fwd-1	gaacaaacagacaatctggtctgtttgtattataaattcgagctcggtacccg
746	Pj5-lacO-NotI-fwd-3	gcggccgcggaattgtgagcggataacaattcaaaaaccgttattgacacaggtggaaa
747	Pj5-lacO-fwd-2	ttattgacacaggtggaaatttagaatatactgggaattgtgagcggataacaattc
748	Pj5-lacO-fwd-1	gtgagcggataacaattcaattcgagctgcgtacccg
749	2Pj5-hcaO-NotI-fwd-3	gcggccgcctacttgatatgtcaggaagcctgatactataaaaaaccgttattgacaca
750	2Pj5-hcaO-fwd-2	aaaaaccgttattgacacaggtggaaatttagaatatactgctacttgatatgtcagga
751	2Pj5-hcaO-fwd-1	ctacttgatatgtcaggaagcctgatactataaattcgagctcggtacccg
752	UpRegab_fwd_KpnI	ggtacctcaccttcagcatgatctgg
753	UpRegab_rev_oe	ggtggcggaatcgagggccatggcgcgagtgtatcaat
754	UpPccbL_PHG_fwd	tacttgatcgtttcattgctatcc
755	UpPcbbL_Chr2_fwd	tgcttgatggtctcgttgct
756	UpPcbbL_fwd-KpnI	ggtaccttcgcgcagcaggaaggt
757	UpPcbbL_rev_oe	ggtggcggaatcgagggccatgcttgtctccttgcgtg
758	PcbbL_fwd_KpnI	ggtacctcgcacttaagggattgcttatac
759	RegA_fwd_NdeI	catatgaccgacaccctcacc
760	RegA_rev_oe	ccgcggtggagatgct
761	RegA_fwd_oe	agcatctccaccgcgg
762	RegA_rev_HindIII	aagctttaccgcgacaccggc
763	cbbRSD_fwd_XbaI	tctagaaataattttgtttaactttaagaaggagatatacatatgtcgtccttcctgcgc
764	cbbR_rev_HindIII	aagctttaccgcgacaccggc
765	C1-Plac_fwd_SpeI	actagtaacgcaattaatgtgagttagctcac
766	C1-T7term_rev_oe	cccttggggcctctaaacgggtcttgaggggttttttgtcaggccacccgccgcccg
767	C2-PCIV1bM_fwd_t7termoe	tttagaggccccaaggggttatgctagtcaacagcgacgaatacagcac
768	rrnbT2terminator_oe_KanR	aatcgatagattgtcgcacctgattgcgtatttagaaaaataaacaa

769	T7terminator_oe_KanR	aatcgatagattgtcgcacctgattgccaaaaaaacccctcaagacccgttta
770	CymR_P fwd SpeI	actagtaattcttgaagacgaaaggg
771	CymR_P oe rev	actcttccttttttcaatctt
772	CymR gen fwd oe	aacattgaaaaaggaagagtatgagtccaaagagaagaac
773	CymR gen T7tt rev 1	cccttggggcctctaaacgggtcttgaggggttttttgctagcgcttgaatttcgcgtac
774	CymR gen T7tt rev 2 SpeI	actagtctagcataacccccttggggcctctaaacg
775	Pt5de20NotIfwd	gcggccgcaaaaaatagtttgacaccctagccgataggctttaagatgaattcgagctcg
776	PT5de33NotIfwd	gcggccgcacttaaaatttatttgcttaaatacttaaacttctgtataatagaattcgag
777	PT5h207NotIfwd	gcggccgcttaaaaaattcatttgctaaacgcttcaaattctcgtataatagaattcgag
778	PT5n25NotIfwd	gcggccgcataaaaaatttatttgctttcaggaaaatttttctgtataatagaattcgag
779	PT5n26NotIfwd	gcggccgcttaaaaatttcagttgcttaatcctacaattcttgatataatagaattcgag
780	PT5f30NotIfwd	gcggccgcttaaaagttttatttgctaaaatgcttaagtttctgtataattgaattcgag
781	PT5k28NotIfwd	gcggccgcgttaaaattgtagttgctaaatgcttaaatacttgctataatagaattcgag
782	PT5k28bNotIfwd	gcggccgctaaagtggttattgacattttcgccgcttaggtatatactagaattcgagct
783	PT5h22bNotIfwd	gcggccgcactaaaaaattgttgacaatagcccagcaatcggtaaaatagaattcgagct
784	PT5Jj5NotIfwd	gcggccgcaaaaaccgttattgacacaggtggaaatttagaatatactgaattcgagctc
785	PT5g25NotIfwd	gcggccgcaaataaaaatttcttgataaaatttttccaatactattataatagaattcgag
786	cmR_NotI_XhoI_fwd	gcggccgcctcgagtcatgacgaataaatacctgtgac
787	PClV1bMfwdSpeI	actagttcaacagcgacgaatacagc
788	Rev cmR SpeI	actagttaactggcctcaggcattt
789	Fwd CmR NotI	gcggccgctcatgacgaataaatacctgtgac
790	lacYoefwd	ctctcgaggaagccaatcatgtactatttaaaaaacacaaacttttgg
791	TermrevSpeI	actagtaaggccatccgtcaggat
792	MOB oriT Rk2 PstI Fwd	aactgcagtcgatcttcgccagcagg
793	MOB oriT Rk2 PstI Rev	aactgcagtcgacatccgccctcac
794	CymR_P fwd SpeI neu	actagtacggatggccttttgcgtt
795	CymR_P oe revneu	actcttccttttcaatgttttatgtaagcagacagttttattgttcatg

796	EstRu_rev_primer_His_EvoRV	gatatctcagtggtggtggtggtg
797	EstRu_fwd_primer_NdeI	ggaattccatatggccctcgattccg
798	Rk2neuspeI	ggactagtagcgtggactcaaggctct
799	Rk2oeneu1	ggatcgtagctaagcatcgtagcgctgccatttttgg
800	Rk2rev PstI	aactgcagaggacgaaaacgaaaagagg
801	Mob rev NotI	ataagaatgcggccgcatggcggcatacgcgat
802	PT5 fwd1 NotI	cgcggccgccaaatcataaaaaatttatttgctttgtgagcgg
803	PT5f fwd2	atttatttgctttgtgagcggataacaattataatagaattcgagctcggtaccc
804	Ptac exp fwd	gcgctcaagcgcgaaggcagccatcggaa
805	pSC101fwdPstI	ctgcagagcttgcgagggtgctactta
806	pSC101revblunt	tgagctgtaacaagtgtctcaggt
807	Rk2fwdSpeI	actagtgatagatctagcgtggactcaagg
808	Rk2oberlap	ctgcagaccagaaccaatcctattca
809	Psafwd1	ctgcagaccagaaccaatcctattcagc
810	Psarev1	actagtctacatactacaacaatttaacagagcca
811	pSarevSpeI	actagtactgtagtatgttgtatgatactacatacta
812	pSafwdPstI	ctgcagaccagaaccaatcctattca
813	Psaseqfwd	gagggaacaacatgcctaagaacaaca
814	Psaseqrev	gcgttggcctggtcaagtcggag
815	cmR fwd oe XhoI	cgggtcttgaggggttttttgctcgagtcatgacgaataaatacctgtgac
816	3´Mcs OE incl NdeI	taactttaagaaggagatatacatatg
817	3´Insert OE excl HindIII	ggctgttttggcggatgagagaagat
818	Pj5-hcaO-NotI-fwd-3	gcggccgcccgcgttggcatgccgacgaagcaaaaaccgttattgacaca
819	Pj5-hcaO-fwd-2	aaaaaccgttattgacacaggtggaaatttagaatatactgccgcgttggcatgccgacg
820	Pj5-hcaO-fwd-1	ccgcgttggcatgccgacgaagcaattcgagctcggtacccg
821	PcmR-SpeI-fwd	actagttcatgacgaataaatacctgtgac
822	PcmR-oe-rev	tttagcttccttagctcctgaaaatct

823	hcaR-oe-fwd	agattttcactaaggaagctaaaatggcaacgtcaggaacgaa
824	hcaR-SpeI-rev	cccttggggcctctaaacgggtcttgaggggtttttgtcagcgcagattgcgcg

a) Primer number of “Team Knallgas” primer list

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